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Regulation of Phospholipase D Pathway

by

Christos Liossis ©

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

Department of Biochemistry

Edmonton, Alberta
Fall 1995

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Sept. 20, 1995

To the two persons I love and respect the most

ΣΤΑΥΡΟΣ και ΑΘΑΝΑΣΙΑ
ΛΙΟΣΗΣ

Abstract

Phospholipase D (PLD) activity in HL 60 and Swiss 3T3 cells was measured in an assay consisting of cell fractions (membranes and/or cytosol) and phosphatidylcholine contained in a mixed phospholipid liposome. In both cell lines, PLD had an absolute requirement for phosphatidylinositol-4,5-bisphosphate. Addition of N-ethylmaleimide and vanadate inhibited this activity. Guanosine 5'-O-(3-thiotriphosphate) (GTPγS) stimulated the membrane-bound PLD only in the presence of cytosolic cofactors, suggesting the involvement of G-proteins. Two recombinant small molecular weight G-proteins, ADP-ribosylation factor (ARF) and RhoA, were prepared, purified and tested for their ability to activate membrane-bound PLD. ARF activated PLD in both cell lines, whereas RhoA stimulated PLD from HL 60 cells. When ARF and RhoA were combined, we observed an additive effect on PLD activity. The cell permeable sphingolipid C_2-ceramide inhibited not only the GTPγS-, but also the ARF-and RhoA- stimulated PLD activity in both cell lines. We conclude that PLD exists as several isoforms which are regulated by several cytosolic and/or membrane factors implicating PLD as an important player in a variety of physiological processes.

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LIST OF ABBREVIATIONS

ACS	aqueous counting scintillant
ARF	ADP-ribosylation factor
ATP	adenosine triphosphate
ADP	adenosine diphosphate
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
Ci	Curie
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DPM	disintegrations per minute
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetate
EGTA	ethyleneglycol bis-(β-aminoethyl ether) -N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
FCS	fetal calf serum
FIG.	figure
g	gram
GAP	GTPase activating protein
GTPβS	guanosine 5'-O-(2-thiodiphosphate)
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
HCl	hydrochloric acid
HRP	horseradish peroxidase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate
IgG	immunoglobulin G
kDa	kilodaltons
KH_2PO_4	potassium dihydrogen phosphate
l	litre
LDH	lactate dehydrogenase
LPA	lysophosphatidic acid
M	molar
$MgCl_2$	magnesium chloride

min	minute
MW	molecular weight
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NEM	N-ethyl maleimide
PA	phospatidic acid
PAGE	polyacrylamide gel electrophoresis
PAP	phosphatidate phosphohydrolase
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidyl ethanolamine
PIP	phosphatidylinositol-4-phosphate
PIP_2	phosphatidylinositol-4,5-biphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PS	phosphatidylserine
rpm	revolutions per minute
sd.	standard deviation
SDS	sodium dodecyl sulfate
tlc	thin layer chromatography
TPA	12-*O* - tetradecanoylphorbol-13-acetate
Tris	tris (hydroxymethyl) aminomethane
Triton X-100	octyl phenoxypolyethoxyethanol

INTRODUCTION

In the last decade, signal transduction has become one of the most fascinating and important aspects of modern biology. Cellular fate, ranging in diversity from proliferation and differentiation to programmed cell death, is the result of a complex and tightly controlled action of signaling molecules including hormones, cytokines, growth factors etc. An impressive number of researchers has set out to understand the exact mechanisms involved in transducing extracellular signals into cellular response.

A plethora of reports have implicated phospholipids as key players in signal transduction processes. Traditionally, phospholipids were thought to serve only as major constituents of biological membranes and were assigned the limited capability of cellular building blocks. Their importance as the source of intracellular second messengers came about during attempts to understand how neurotransmitters, hormones etc., conferred their effects on cells. It was then realized that the breakdown of specific phospholipids, due to the action of phospholipases coupled to cell surface receptors, gave rise to biologically active products which were able to modulate the activity of target intracellular macromolecules and enzymes, eliciting a specific cellular response.

The Introduction will deal specifically with phospholipase D (PLD) providing an overview of its occurrence, regulation and physiological importance and will touch upon the other two best-characterized phospholipases C, and A_2.

GENERAL DESCRIPTION AND IMPORTANCE OF PLD IN SIGNAL TRANSDUCTION

PLD (E.C. 3.1.4.4) catalyzes the hydrolysis of phospholipids by attacking the terminal phosphodiester bond, giving rise to phosphatidate (PA) and the free polar head group according to the following phosphatidyl transfer reaction:

phosphatidyl-O-choline + H-OH ➔ phosphatidate + choline

The enzyme is also capable of catalyzing a transphosphatidylation reaction whereby the phosphatidyl group of phospholipids is transferred to a primary alcohol such as ethanol or butan-1-ol:

phosphatidyl-O-R + R_1-OH➔ phosphatidyl-O-R_1 + R-OH
(when $R_1 = C_n H_{2n+1}$)

Researchers have taken advantage of this unique ability of PLD to produce phosphatidyl alcohols in studies involving activation of PLD. Unlike PA, the natural product of phospholipid hydrolysis by PLD, phosphatidylethanols are metabolized extremely slowly and accumulate in the cell upon activation of the enzyme, as was the case in HL-60 cells stimulated by a chemotactic peptide in the presence of ethanol (Pai *et al.* 1988). The agonist-stimulated increase of phosphatidylalcohols is now widely accepted as a measure and indication of elevated PLD activity and has been used to monitor the effect of a variety of signaling molecules on PLD in diverse cell types.

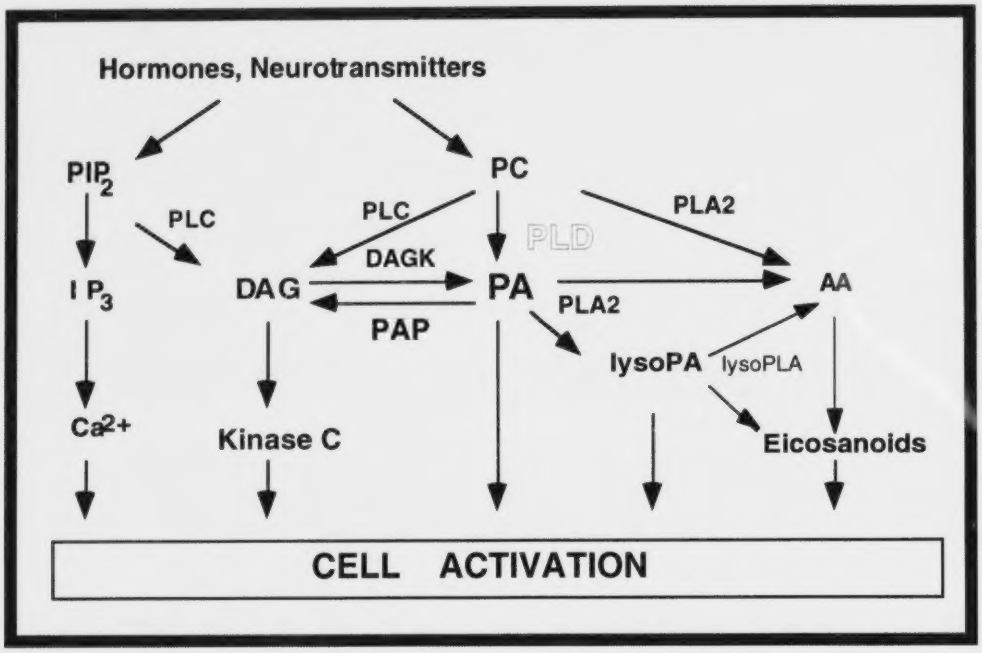


Figure adapted from Dr. D. N. Brindley

Figure I.1 Signal transduction pathways involving major phospholipases. Abbreviations used in this table: phosphatidyl inositol-4,5-bisphosphate (PIP_2), 1,4,5-inositol triphosphate (IP_3), phospholipase C (PLC), diacylglycerol (DAG), protein kinase C (kinase C), phosphatidylcholine (PC), phosphatidate (PA), diacylglycerol kinase (DAGK), phosphatidate phosphohydrolase (PAP), phospholipase A_2 (PLA2), arachidonic acid (AA), lysophosphatidate (lysoPA).

Table 1 illustrates the action of several phospholipases and their products as well as other enzymes involved in signal transduction. As it was alluded to earlier, the primary product of PLD activity is PA. This lipid can have one of three different metabolic fates. Phosphatidate phosphohydrolase (PAP) can convert PA to DAG, explaining the “late” rise of DAG in the biphasic properties of agonist-stimulated DAG production and the sustained activation of PKC, which in turn can stimulate cell division. The “early” rise of DAG due to agonist stimulation is brought about by the action of different isoforms of PLC on inositol phospholipids and/or phosphatidylcholine. Breakdown of inositol phospholipids leads to a rise in the level of IP_3, which stimulates the release of Ca^{2+} from intracellular stores, leading to activation of the Ca^{2+} sensitive isoforms of PKC. Phospholipase A_2 action on PA results in the production of LPA (lysophosphatidate), whereas PLA_2 action on PC leads to the release of arachidonic acid which induces inflamatory responses and is the precursor of eicosanoids. Lastly, PA can also be routed for re-synthesis of certain phospholipids in the form of CDP-DAG.

A number of *in vitro* studies have shown that PA can modulate a variety of regulatory enzymes although no evidence exists for *in situ* action of PA. Tsai *et al.* (1989) have shown that PA inhibits RasGAP (Ras GTPase-activating protein) thus prolonging the effects of the activated form of the enzyme i.e. when Ras is bound to GTP. In addition PA stimulated PI-PLC-γ (Jones *et al.*, 1993), NADPH oxidase of pig neutrophils (Bellavite *et al.*, 1988), a Ser/Thr kinase (Bocckino *et al.*, 1991), n-Chimaerin *rac* -GAP (Ahmed *et al.*, 1993), protein kinase C_η (Ballas *et al.*, 1993), and protein tyrosine phosphatase 1C (Zhao *et al.*, 1993). It also exerts an inhibitory effect on adenylate cyclase in a pertussis-toxin sensitive manner, which implicates the involvement of the inhibitory G-protein (G_i) of adenylate cyclase (Murayama *et al.*, 1987). PA and its metabolic product LPA can act as mitogens when added exogenously in cultured fibroblasts, conferring their mitogenic action through a G-protein-coupled receptor (van der Blend *et al.*, 1992). Addition of PA and LPA can also activate PLD, resulting in generation of intracellular PA (Martin *et al.*, 1993). In extracts of Balb/c 3T3 cells, the activity of PLD and the mass of PA correlated with the mitogenic activity of PDGF (Fukami *et al.*, 1992). Increased PA mass due to enhanced phospholipid turnover has also been observed in *ras* -transformed fibroblasts (Martin *et al.* 1995, submitted).

PA and LPA have also been linked to cell-shape changes and differentiation. Ha *et al.*, (1994) suggested that exogenously added LPA to IIC9 fibroblasts increased PLD activity in a pertussis-toxin sensitive manner. The resulting increase of intracellular PA induced an increase in F-actin polymerization. The same workers provided evidence suggesting that the changes in cell morphology of IIC9 fibroblasts in response to α-thrombin are mediated by increases in PA levels derived from PC. DAG and PKC had inhibitory effects on the PA-induced actin polymerization (Ha *et al.*, 1993). LPA and PA also stimulated the stress fiber formation in Swiss 3T3 cells (Ridley *et al.*, 1992). The response to LPA was inhibited by *C. botulinum* toxin, which specifically inhibits small molecular weight G-proteins of the Rho family. The same effect was produced with micro-injection of Rho that had already been ADP-ribosylated, indicating that the effects of LPA on actin stress fiber formation are mediated by actions of Rho on the cytoskeleton. LPA is found in small quantities in serum and can be released into the plasma from thrombin-activated platelets, ascribing a physiological role for this lysophospholipid (Eichholtz *et al.*, 1993).

In summary, there is strong evidence to suggest that the products stemming from PLD activation-namely PA and LPA-play important roles in a variety of cellular processes by modulating the activity of several key regulatory enzymes. In light of such compelling information, PLD is considered a very important enzyme since its metabolic product, PA is strategically placed in the crossroads of cellular function and phospholipid biosynthesis.

OCCURRENCE AND CHARACTERIZATION OF PLD

PLD occurs extensively in the plant kingdom and has been purified to homogeneity from cabbage (Abouslaham *et al.* 1993). The mammalian counterpart still evades purification to completion although there are a number of reports describing partial purifications. In a series of reports (1974-1983) Kanfer *et al.* were the first ones to characterize mammalian PLD found in rat brain membranes. They reported a K_m value of about 0.9 mM for both PC and PE. The enzyme was slightly stimulated by low concentrations of Ca^{2+} and Mg^{2+} and had a pH optimum of 6. In 1990, Chalifa *et al.* (1990) reported a neutral PLD (pH optimum of 7.2) found in rat brain synaptosomes that was strongly stimulated by oleate (4 mM). In addition, Kanoh *et al.* (1991) showed that this PLD, from rat brain, can be stimulated by 0.1-0.2% Triton X-100 and is inhibited by Ca^{2+} and Mg^{2+}.

A number of reports have dealt with the substrate specificity of PLD (Mohn *et al.* 1992, Daniel *et al.* 1993). The neutral brain PLD preferred acyl- and alkyl -PC and the enzyme activity was slightly affected by the nature of fatty acid in the *sn* -2 position. PLD from Madin-Darby canine kidney cells also preferred PC as substrate. In the same study, the majority of phosphatidylethanol produced upon phorbol ester stimulation of PLD was highly enriched in oleic acid in the *sn* -2 position. Horwitz and Davis (1993) conclusively showed that PC is the best substrate in rat brain microsomes by assaying phosphatidylbutanol synthesis using $[^3H]$butanol as labelled substrate. All the reports mentioned above dealt with membrane bound PLD. Wang *et al.* (1991) reported a 20-fold purification of a cytosolic PLD from bovine lung. This enzyme exhibited a different substrate preference than its membrane counterpart by hydrolyzing PE and PI and PC and was unaffected by Mg^{2+}.

In 1994, Okamura and Yamashita purified PLD (2200-fold) from pig lung microsomes. The enzyme had an apparent molecular weight of 190 kDa, a pH optimum of 6.6 and metabolized dipalmitoyl-PC, over LPC, PE, and PI, with a K_m of about 0.8 mM. Higher concentrations of Ca^{2+} and Mg^{2+} (1 and 2 mM, respectively) were stimulatory, whereas lower concentrations of the same divalent ions had no effect. Unsaturated fatty acids (oleate, linoleate and arachidonate at 2 mM) markedly stimulated the purified enzyme, up to 5-fold in the case of arachidonate, with saturated fatty acids being completely ineffective. Recently, Brown *et al.* (1995) described the purification (5,000-10,000-fold) of PLD from porcine brain membranes. This enzyme was activated by the small molecular weight G protein ARF (G protein regulation of PLD as well as the implications of the purification of ARF-sensitive PLD will be described later in the relevant section in the Introduction). Hydrodynamic analysis indicated that the enzyme had an apparent molecular mass of 95 kDa.

Overall, the above reports provide strong evidence that PLD exists as multiple isoforms that exhibit different substrate specificities and subcellular localizations in a tissue-specific manner. Exact characterization of the multiple isoforms of PLD is foreseeable in the near future now that purification of the enzyme from different sources has being accomplished.

REGULATION OF PLD ACTIVATION

ROLE OF PKC AS AN UPSTREAM ACTIVATOR OF PLD

Receptor-mediated activation of PLD has been shown using a variety of agonists ranging from vasopressin to EGF and LPA (for a complete list refer to Liscovitch *et al.*, 1994). Most of these agonists are known to confer their actions by activating a PI-specific PLC. PI-PLC hydrolyzes PI to inositol-1,4,5-triphosphate and 1,2-*sn* -diacylglycerol whose targets are intracellular Ca^{2+} organelles and PKC respectively (Berridge 1993, and Nishizuka 1992, respectively). Since activation of PLD using these agonists was usually seen after inositol-1,4,5-triphosphate production, one of the pathways leading to stimulation of PLD is thought to involve the activation cascade of PI-PLC to PKC to PLD. Further support that PKC controls the activation of PLD came from reports that utilized the tumor promoting factor PMA, a DAG analog, that activates PKC in the absence of any receptor-mediated effect (Exton, 1990, Billah and Anthes, 1990). In addition, selective inhibitors of PKC such as the competitive ATP-binding site inhibitor Ro-21-82220 (Cook *et al.*, 1991) and the bisindoylmaleimide GF109203X (Gustavsson *et al.*, 1994), as well as down-regulation of PKC by prolonged phorbol ester treatment (Ben-Av *et al.*, 1993), block the activation of PKC and abolish the accumulation of phosphatidylalcohols in response to PMA.

Many groups have attempted to answer the question of which isoform(s) of PKC is responsible for the activation of PLD, but so far the available evidence is contradictory and could only be explained due to expression of several isoforms of PLD in a tissue-dependent manner. Studies involving overexpression of PKCα in Swiss 3T3 fibroblasts (Eldar *et al.*, 1993) and PKCβ-1 in rat fibroblasts (Pachter *et al.*, 1992) indicated that both basal and stimulated PLD activity were elevated following exposure to PDGF, endothelin-1, thrombin, or PMA. Yet, since the dose response curves of stimulated PLD to PDGF and

PMA did not shift they concluded that the PKCα isoform could play a role in the regulation of the expression of PLD rather than in its acute stimulation by PMA. On the other hand, Conricode *et al.* (1994) studied the regulation of PLD by different PKC isozymes in Chinese hamster lung fibroblasts (CCL39 cells). PKCα isolated from rat brain was the most potent in activating PLD, when stimulated by PMA, with β and γ exhibiting moderate or no potency at all, respectively. In the same report recombinant isoforms of PKC (δ, ε, ζ) were also tested but were shown to be ineffective. Since immunoblot analysis of the soluble fraction of CCL39 cells indicated the presence of α and ζ PKC isoforms these authors concluded that PKCα is responsible for the activation of PLD in their cell system. Additional evidence by Balboa *et al.* (1994), who selectively reduced the levels of either PKCα or PKCβ in MDCK cells using antisense oligonucleotides, has implicated PKCα but not PKCβ in the activation of PLD. Finally Jones *et al.* (1995), found that C_2-ceramide inhibited the diC_8-mediated (in HEL-37 cells) or the bradykinin mediated (in SF3155 cells) activation of PLD by selectively inhibiting PKCα translocation to particulate material.

The mechanism by which PKC activates PLD has sparked even more contradiction. Several lines of evidence indicate that the activation is dependent on protein phosphorylation. Certainly the abolishment of PLD activity in the presence of Ro-31-8220, a competitive ATP-binding site inhibitor of PKC, adds to this notion. Furthermore, two independent reports by Olson *et al.* (1991), and Kusner *et al.* (1993), showed that in both human neutrophils and promonocytic leukocytes, PMA activation of PLD was dependent on Mg^{++}ATP and required the addition of cytosol in permeabilized cells. Conricode *et al.* (1992) observed a dose dependent effect on both PMA and PKC stimulation of CCL39 membrane PLD. The stimulation was observed in the absence of ATP and was even sustained when apyrase, an ATPase, was included. The presence of apyrase completely inhibits both the phosphorylation of exogenously added histone, a substrate of PKC, and PKC autophosphorylation. These results indicate that PKC activates PLD by non-phosphorylating mechanisms that could involve protein-protein interactions.

The evidence provided in this section further suggests that there are several isoforms of PLD. Isolation and characterization of all these will be required before a definite explanation as to which isoform of PKC and by which mechanism activates one or multiple isoforms of PLD.

ROLE OF GROWTH FACTORS AND TYROSINE PHOSPHORYLATION IN PLD ACTIVATION

The PLD activation through PKC described above was mostly due to phosphoinositide turnover mediated by agonists whose receptors are coupled to G-proteins. Another class of agonists includes growth factors such as EGF, bFGF,and PDGF whose receptors are tyrosine kinases. Cook *et al.* (1992) showed that EGF-induced PLD activation in Swiss 3T3 cells is independent of PKC because this growth factor does not induce the hydrolysis of phosphoinositides. This report was recently challenged by Yeo *et al.* (1995) who showed that the activation of PLD by EGF in the same cell line was inhibited in a dose dependent manner by two PKC inhibitors, Ro-31-8220 and GF109203X. Furthermore they proved that in the presence of Ro-31-8220, EGF can stimulate [^{3}H]DAG formation from cells pre-incubated with [^{3}H]arachidonate, which labels mainly the PI pool, but not from cells pre-incubated with [^{3}H]myristate, which labels the PC pool. Surprisingly, in the same report Ro-31-8220 blocked PLD activation although it promoted translocation of PKC$_\alpha$ in the presence of EGF. This finding contradicts the evidence they provided in an earlier report (Conricode *et al.*, 1992) where PLD from CCL39 membranes was activated by PKC with no requirement of catalytic activity of PKC (phosphorylation-independent mechanism).

A strong correlation between tyrosine phosphorylation and PLD activation has been established by Bourgoin *et al.*, (1992). Treatment of permeabilized HL 60 cells with vanadyl hydroperoxide, a P-tyrosine phosphatase inhibitor, lead to an ATP and Mg^{2+} dependent increase of PA or phosphatidylethanol. Moreover, in Balb/c 3T3 cells transformed with v-Src, a constitutively active non-receptor-associated tyrosine kinase, Song *et al.* (1991) observed an increase in transphosphatidylation activity, indicating that PLD can be activated by a tyrosine kinase. The same group (Jiang *et al.*, 1995) have shown that the reported increase of PLD activity due to v-Src is dependent upon GTP and cytosol. These workers observed an inhibition of PLD activity using a neutralizing monoclonal antibody against Ras and a dominant-negative Ras mutant in the v-Src transformed cell line. These findings demonstrate that the monomeric G protein Ras is mediating the activation of PLD by v-Src.

REGULATION OF PLD ACTIVITY BY G-PROTEINS

The involvement of G-proteins in the regulation of PLD has been implicated by the use of chemical agents that either render G-proteins active, such as GTPγS (for monomeric and heterotrimeric G-proteins), and $AlF_4{}^-$ ions (heterotrimeric G-proteins), or inactive such as GDPβS (for monomeric and heterotrimeric G-proteins), and pertussis toxin (ADP-ribosylating agent of G_o and G_i families of G proteins thus uncoupling them from their receptors). In rabbit neutrophils the activation of PLD by the chemotactic factor fMet-Leu-Phe was mediated by a pertussis toxin sensitive G-protein (Kanaho *et al.*,1991); in contrast, insensitivity to this toxin has been observed in carbachol-activated PLD from rat PC12 cells. As early as 1987, Bocckino *et al.* showed that GTPγS could stimulate PLD in rat hepatocytes. The role of the divalent ions Mg^{2+} and Ca^{2+} in the guanine nucleotide activation of PLD has been studied extensively and has given variable results. Xie *et al.* (1991) showed a Mg^{2+} requirement for GTPγS activation of PLD in electropermeabilized HL 60 cells. PLD from permeabilized NG108-15 cells was active at subnanomolar concentrations of Ca^{2+}, but higher concentrations were inhibitory (Liscovitch *et al.*, 1991). In addition, GTPγS activation of PLD from HL 60 cells occurred in the absence of Ca^{2+} (Xie *et al.*, 1991). Contrary to this observation, Geny *et al.* (1992) showed that increasing the concentration of Ca^{2+} (from 100 nM to 10 μM) alone can stimulate PLD activity in HL 60 cells. On the other hand, MgATP is not required for GTPγS -dependent PLD activity in these cells (Geny *et al.*, 1992).

Initial evidence that both cytosol and membranes were required for reconstitution of GTPγS-dependent PLD activity was obtained in studies utilizing permeabilized granulocytes (Olson *et al.*, 1991, Anthes *et al.*, 1991). The dependence on cytosol was further demonstrated in studies where depletion of cytosolic components resulted in the absence of GTPγS stimulation of PLD activity (Geny *et al.*, 1992).

Two groups have independently identified and characterized the cytosolic factor required for this stimulation as the low molecular weight GTP-binding protein, ADP-ribosylation factor, ARF (Brown *et al.*, 1993, and Cockcroft *et al.*, 1994). To aid in the purification protocol of this factor, Brown *et al.* (1993) developed a novel assay for measuring PLD activation in which a mixed phospholipid liposome containing PE, PI-4,5-P_2, and PC as the substrate was presented to the enzyme. The presence of PI-4,5-P_2 was found to be absolutely required for observing any kind of PLD activity in this system. PI-4,5-P_2 has been shown to be a potent activator of partially purified rat brain PLD with an EC_{50} <1 mol% (Liscovitch *et al.*, 1994). Moreover, blocking *in vitro* PI-4,5-P_2 synthesis in permeabilized U937 cells resulted in a almost complete inhibition of the GTPγS-dependent PLD activity (Pertile *et al.*, 1995). Using this assay system these workers purified the cytosolic factor from bovine brain cytosol. The purified protein had a molecular weight of 21 kDa and its amino acid sequence was identical to that of ARF1 and ARF3 from bovine and human cDNA respectively. Cockcroft arrived at the same result using HL 60 cells prelabelled with [^{3}H]alkyl-lysoPC or [^{3}H]choline as sources of substrate for PLD. Initially, ARF was identified as the protein cofactor required for cholera toxin-induced ADP-ribosylation of the α subunit of G_s, the stimulatory subunit of adenylyl cyclase (Kahn *et al.*, 1984). Recent advances in this field with the use of amino-terminal deletion mutants have shown that ARF facilitates the ribosylation of G proteins by binding both G_s with its N-terminal domain (Randazzo *et al.*, 1994), and the cholera toxin with a domain lying within its carboxy-terminal two thirds of the protein (Zhang *et al.*, 1995).

ARF proteins (ARF1 through ARF6) are members of the Ras superfamily of regulatory GTP-binding proteins. They are structurally and functionally conserved and seem to be ubiquitously expressed in a diversity of cells ranging from yeast to bovine and human (Tsuchiya *et al.*, 1991). Their role in protein transport was demonstrated in *S. cerevisiae* where ARF mutants caused secretory defects of the protein invertase and were synthetically lethal with a subset of genes known to be secretory defective, i.e. SEC

(sec21-1 sec7-1) (Stearns *et al.*, 1990). In the same study, ARF was found at the cytosolic side of *cis* -Golgi structures, implicating them as sites of ARF-mediated effects. Another important function of ARF is its association with clathrin- and non-clathrin- coated vesicles assisting in coatomer association with target membranes, as well as the assembly of coat proteins (Rothman *et al.*, 1992). ARF has also been shown to influence membrane trafficking by inhibiting intra-Golgi transport, endosome, and nuclear vesicle fusion in a GTPγS-dependent fashion (Taylor *et al.*, 1992, Boman *et al.*, 1992). In light of the information that ARF is also an upstream regulator of PLD, Kahn *et al.* (1993) suggested that the PA produced by ARF-dependent-PLD during membrane trafficking could either promote fusion events, give rise to DAG, or act as a "feedback" switch by acting on effectors of ARF, i.e. ARF GAP.

ARF proteins also exhibit some fascinating biochemical properties. The active form of ARF (GTP bound form) associates stably with phospholipid micelles and allows for the redistribution of the protein from the cytosol to the membranes (Kahn *et al.*, 1991). Myristoylation of the N-terminal glycine is required for GTP-dependent binding of ARF to the membranes. Brown *et al.* (1993) found that the myristoylated recombinant ARF1 was a better activator of PLD activity than was the non-myristoylated form. The "loading" of ARF with GTP is dependent on the presence of phospholipids (Kahn *et al.*, 1986). Recently Franco *et al.* (1995) showed that myristoylation decreases the rate of GTP dissociation 10-fold. In the same study, myristoylated GDP-bound ARF interacted with phospholipids exclusively through its myristate residue; in addition to its myristate, GTP-bound myristoylated ARF interacted with the same phospholipids also through its N-terminal hydrophobic residues. The N-terminal region of ARF is not only the "business" end of the protein but also, when myristoylated, a sensor of phospholipid availability. In the absence of phospholipids myristoylated ARF has higher affinity for GDP; in the presence of phospholipids the protein has greater affinity for GTP. It is therefore conceivable that myristoylation allows for the concerted effect of ARF-activation upon phospholipid and GTP binding (Randazzo *et al.*, 1995). Although ARF is classified as a G-protein, it has no intrinsic GTPase activity (Randazzo *et al.*, 1994). In the same report the authors characterized a specific ARF-GAP from bovine brain extracts. The presence of PI-4,5-P_2 stimulated ARF-GAP activity 8-fold. This activity could be stimulated further by phosphatidylalcohols and inhibited by PC. PA also stimulated ARF-GAP by 5-fold and reduced the PI-4,5-P_2 concentration needed for ARF-GAP stimulation 6-fold, implying the existence of negative loop feedback resulting from acute raises of PA due to the action of PLD. Lastly, ARF also contains a highly specific PI-4,5-P_2 binding site. The presence of PI-4,5-P_2 enhances the binding of GTP to ARF by promoting the dissociation of GDP, and stabilizes the protein to its active (GTP-bound) form (Terui *et al.*, 1994).

Increasing evidence has also implicated Rho as an upstream regulator of PLD. Rho is also a small molecular weight G-protein that belongs to the Ras superfamily. Members of the Rho family are involved in NADPH oxidase activation, membrane ruffling, and actin polymerization (Ridley *et al.*, 1992). Bowman *et al.* (1993) found that in HL 60 cells, PLD could be activated in the presence of GTPγS by a membrane bound protein. Heterotrimeric G-proteins were excluded due to absence of PLD activation in the presence of AlF^-_4. Addition of RhoGDI (an inhibitor of GDP dissociation from Rho) abolished this activity, while RhoGDS promoted the stimulation of PLD, indicating that indeed Rho was involved in PLD regulation. Malcolm *et al.* (1994) arrived at the same conclusion after extraction of RhoA from rat liver membranes with RhoGDI diminished GTPγS-dependent PLD activity. The combination of ARF and RhoA showed a synergistic effect in the activation of PLD in HL 60 cells (Siddiqi *et al.*, 1995). In the same study, the authors provided data that suggest that ARF regulates a cytosolic isoform of PLD, whereas RhoA regulates the membrane-bound PLD.

It has become apparent from this overview that PLD regulation is complex and multi-faceted. PLD exists in a number of isoforms and is found in different compartments within the cellular infrastructure. They all exhibit different sensitivities to cytosolic and membrane-bound regulatory enzymes (PKC, ARF, RhoA, etc.). The importance of this highly controlled enzyme, and its physiological function, will become evident once integration of the regulatory pathways that modulate its activity is accomplished. PLD will find its place in one or more signal transduction events whose mechanisms can be traced step by step, from the initial agonist interaction with a receptor to the ultimate cellular response.

THE Mg^{2+}-DEPENDENT, N-ETHYLMALEIMIDE SENSITIVE PHOSPHATIDATE PHOSPHOHYDROLASE 1

Phosphatidate phosphohydrolase 1 (PAP-1) is one of the two members of phosphohydrolases (EC 3.1.3.4) occurring in a variety of tissues . The activity of PAP-1 can be easily distinguished from the one arising from PAP-2 with the use of NEM, a sulphydryl-group alkylating agent (see also relevant section in Materials and Methods) (Martin *et al.*, 1987). Unlike PAP-2, which is an integral membrane protein (Jamal *et al.*, 1991), PAP-1 is found in the cytosol but has the ability to translocate to the endoplasmic reticulum, where it is involved in glycerolipid synthesis (Brindley, 1984). This enzyme, which has been extensively studied in liver and adipose tissue, catalyzes the conversion of PA to DAG with the release of inorganic phosphate. In rat hepatocytes PAP-1 translocates to membranes in response to high levels of oleate and other long chain fatty acids, fatty-acyl CoA esters and PA, indicating that PAP-1 is attracted to membranes that possess increased negative charge (Gomez-Munoz *et al.*, 1992). This translocation also correlates with an increase of PAP-1 activity. In line with this observation, Martin *et al.* (1986) have shown that chlorpromazine, an amphiphilic amine that decreases the negative charge of membranes, displaces PAP-1 from hepatocyte membranes. Insulin facilitates the translocation to the membranes at lower fatty acid concentrations whereas glucagon and cAMP counteract this effect (Pittner *et al.*, 1985), suggesting not only that regulation of PAP-1 activity is compatible to that observed in gluconeogenic enzymes, but also that translocation of PAP-1 could be regulated by a phosphorylation mechanism.

The physiological role of PAP-1 in the liver is to provide DAG necessary for triacylglycerol synthesis, especially when free fatty acid and fatty acid CoA ester concentrations in the blood are increased (Martin *et al.*, 1994). In addition, PAP-1 could also be involved in signal transduction by actively sensing rises of PA, its physiological substrate, due to agonist-stimulated PLD. Thus PAP-1 can act as a sensitive switch that converts increased levels of PA into another bioactive second messenger, DAG.

MATERIALS

Gibco BRL, Grand Island N. Y.

Phosphate buffered saline (PBS), penicillin, streptomycin, Fetal Calf Serum (FCS), trypsin-EDTA, L-glutamine, N,N,N',N'-Tetramethylethylenediamine (TEMED).

Sigma, St. Louis MO.

Dulbecco's Modified Eagle's Medium (DMEM), bovine serum albumin, bovine serum albumin (fatty acid free), dithiothreitol (DTT), sucrose, 4-(2-hydroxylethyl)-1-piperazineethane sulfonic acid (PIPES), [Tris (hydroxymethyl) aminomethane] hydrochloride, potassium hydroxide, digitonin, sodium pyruvate, L-α-phosphatidylcholine from bovine brain, L-α-phosphatidylethanolamine, dioleoyl (C18:1,[cis]-9) L-α-phosphatidylinositol 4, 5-bisphosphate, L-α-phosphatidylinositol 4-monophosphate, adenosine triphosphate, ultra-pure methanol, ultra-pure chloroform, magnesium chloride, calcium chloride, potassium chloride, maleic acid, EGTA, EDTA, *N*-ethyl-maleimide, Dowex 50X8 200 mesh.

Boehringer Mannheim, W. GERMANY

Guanine diphosphate γ-thiophosphate, guanine monophosphate β-thiophosphate, NADH

Amersham International, Amersham, U. K.

[^{3}H]choline chloride, [^{3}H]phosphatidate, phosphatidyl[^{3}H]choline, enhanced chemiluminescence kit (ECL)

Corning, Oneonta, N.Y.

Various tissue culture plates.

Pharmacia

gel filtration apparatus and fplc

Biorad

gel, protein transfer apparatus, and protein assay

METHODS

CELL CULTURE PROTOCOLS

RAT-1, 2 AND *RAS* -TRANSFORMED FIBROBLASTS

The Rat fibroblasts were cultured in high glucose DMEM supplemented with 10% (v/v) fetal bovine serum, 10 u/ml penicillin and 10 μg/ml streptomycin, under a constant atmosphere of 5% CO_2 in air at 37 °C, and were passaged every three days. For the PAP-1 experiments mildly trypsinized Rat 2 and *ras* -transformed fibroblasts were plated at 1.2×10^6 cells per 10 cm tissue culture dish, or at 7.2×10^5 cells per 60 mm tissue culture dish.

SWISS 3T3 CELLS

Swiss 3T3 fibroblasts were grown in DMEM-GLUTAMAX medium supplemented with 10% (v/v) fetal bovine serum, under a constant atmosphere of 5% CO_2 in air at 37 °C, and at 95% humidity. Cells were routinely passaged under subconfluent conditions. For *in vitro* PLD experiments cells were grown to 90% confluence. The cells were then washed three times in ice-cold PBS and harvested by scraping using solution A (137 mM NaCl, 8.1mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 1mM DTT, 0.1 mM PMSF, 10μg/ml leupeptin, and 1mM Benzamidine pH 7.5) and the collected material was sonicated for 10 seconds. The cytosol was then separated from the membranes by centrifuging at >100000 x g for 30 minutes using a bench top ultracentrifuge. The pellet was resuspended in solution A by sonication. Both the supernatant and membrane fraction were frozen at -70 °C until they were assayed.

HL 60 CELLS

HL 60 cells were grown in RPMI medium supplemented with 15% heat-inactivated fetal bovine serum, under a constant atmosphere of 5% CO_2 in air at 37 °C, and at 95% humidity. For differentiation studies, cells were seeded at a higher density and were allowed to grow in medium containing 1.3% DMSO for three to five days to ensure complete granulocytic differentiation. Both undifferentiated and differentiated cells were collected by centrifugation at 800 rpm in a bench top Beckman centrifuge. They were then washed five times with 3 to 4 vol of solution A and sonicated for 10 seconds in 1 vol of solution A. The HL 60 membranes were separated from the cytosol as described above.

ASSAY PROTOCOLS

PROTEIN CONCENTRATION

The protein concentrations were measured by the Bio-Rad assay method. The samples were assayed neat or diluted 1:10, 1:20, or 1:40. Ten µl of sample was transferred to individual wells on a 96 well micro-titre plate. To each well 200 µl of a 1:5 diluted dye reagent was added and the mixture was left standing at room temperature for 5 minutes to ensure color development. The change of absorbance at 595 nm was detected using a SLT Labinstruments plate reader.

STIMULATION OF RAT FIBROBLASTS WITH OLEATE

Rat 2 and *ras* -transformed fibroblasts were plated at a density of 1.2 x 10^6 cells per 10 cm tissue culture plate. At days 1, 2, and 3 cells were washed twice with ice-cold PBS. The cells were then made quiescent by incubation with DMEM medium containing 0.2% BSA that was essentially fatty acid free. Prior to the stimulation, the medium was aspirated and medium containing 0.5, 0.75, or 1 mM oleate in 0.2% BSA was added for a 15-minute incubation at 37 °C.

PERMEABILIZATION OF FIBROBLASTS

To characterize the permeabilization of fibroblasts using digitonin, cells grown on 10 cm plastic plates were washed twice in ice-cold PBS, and 2 ml of a buffer containing 10 mM HEPES/KOH, pH 7.4, 1 mM DTT, or 0.25 mM sucrose, 10 mM HEPES/KOH, pH 7.4, 1 mM DTT, and 0 to 1 mg/ml digitonin was added. The incubations were performed at 4 °C and the supernatant was collected at various times of up to 15 minutes. The permeabilized cell ghosts were collected by scraping with an equal volume of digitonin-free buffer.

For the experiments involving localization of PAP1, cultured normal and transformed fibroblasts grown on 60 mm plastic dishes were permeabilized with 1 ml of a buffer containing 10 mM HEPES/KOH, pH 7.4, 1 mM DTT, and 0.25 mg/ml digitonin at 4 °C for 4 minutes. The supernatant was collected and the cell ghosts were scraped with 1 ml of digitonin free-buffer.

LACTATE DEHYDROGENASE ASSAY (LDH)

A known volume of a sample (10 to 30 μl) was transferred to individual wells on a 96 well micro-titre plate and the plate was placed into a SLT Labinstruments plate reader. The reaction was started by the addition of 200 μl of assay buffer containing the following reagents: 175mM Tris, pH 7.4, 0.15 mg/ml sodium pyruvate, 0.24 mg/ml NADH. The presence of LDH was detected by the decrease in absorbance due to the conversion of NADH to NAD^+ which was measured spectrophotometrically every 30 second at 340 nm using the kinetic program of the microplate reader. The presence of LDH was estimated as μmole NAD^+/ml per min.

PREPARATION OF [^{3}H]PHOSPHATIDATE SUBSTRATE FOR PAP-1 ASSAY

The substrate was prepared according to Martin *et al.* 1993 by sonicating 3.33 mM [^{3}H]phosphatidate and 2.22 mM phosphatidylcholine in 5.56 mM EGTA and 5.56 mM EDTA. To every 9 vol of this mixture was added 1 vol of 100 mg/ml of fatty acid poor BSA.

PHOSPHATIDATE PHOSPHOHYDROLASE -1

The accurate way to distinguish between PAP-1 and PAP-2 activities in fibroblasts is by using NEM. This reagent specifically inhibits the cytosolic PAP-1 but not the membrane-bound PAP-2. Although the assay conditions reported below have been designed specifically for optimum activity of PAP-1, PAP-2 is suboptimally active and contributes to the overall activity measured. In order to correct for that contribution, parallel reactions to those measuring PAP-1 were run with the addition of 3.4 mg/ml NEM. The difference between the two activities is a true measure of PAP-1 activity.

For the PAP-1 activity measurements, samples were assayed at three different protein concentrations according to the following protocol: a known volume of sample was mixed with a buffer containing 100 mM Tris/maleate, pH 6.5, 1mM DTT, 1.5 mM $MgCl_2$. The volume was adjusted to 80 µl and the reaction was started with the addition of 20 µl of a $[^3H]$phosphatidate/phosphatidylcholine mixed liposome substrate. The reaction was stopped after 60 minutes by adding 2 ml of chloroform/methanol (19:1, v/v) containing 0.08% of olive oil as a carrier. Unreacted $[^3H]$phosphatidate was removed with the addition of alumina and a known volume of the chloroform phase containing the reaction product, $[^3H]$diacylglycerol, was removed, dried and counted by a liquid scintillation technique. The activity is reported as nmol of diacylglycerol produced/min/mg of protein.

PHOSPHOLIPASE D

Cells were harvested, as described earlier, in solution A. Membrane fractions and cytosol were collected after sonicating and centrifuging at 100000 x g for 30 minutes. The protein content was measured and the samples diluted such that 20-25 µg of crude membrane protein and, when required, 20-25 µg of cytosol protein were employed in each assay. All the assays were performed in silica-based glass test tubes to avoid attachment of either protein or lipid to the test tube walls. The sample was pre-mixed in a volume of 30µl with 30 µM GTPγS dissolved in a solution of 30 mM $MgCl_2$, 20 mM $CaCl_2$ plus 400mM NaCl plus ARF, cytosol, or any other addition, at 4° C.

The substrate was freshly prepared for each assay. The lipids forming the substrate liposome were dried down in a glass tube and taken up in a resuspension buffer containing

125 mM HEPES, 7.5mM EGTA, 200 mM KCl, 2.5 mM DTT. The solution was then sonicated at a high setting for 10 seconds using a probe sonicator, and an aliquot was counted by liquid scintillation to ensure that all the radioactivity was in solution and the lipids were not attached to the glass tube. The final concentrations of the lipids forming the liposome were as follows:

LIPID	**CONCENTRATION (µM)**
Phosphatidyl serine or Phosphatidyl inositol bisphosphate	12
Phosphatidyl ethanolamine	137.6
Phosphatidyl choline	8.6 (Specific Activity about 26 Ci/mol or 60000 dpm per assay)

The reaction was started by the addition of 20 µl of the substrate liposome and the incubation was performed in a water bath at 37 °C for 60 minutes. At the end of this time 750 µl of methanol/chloroform (2/1, v/v) was added to stop the reaction. Next 250 µl of chloroform and 400 µl of water were added to separate the two phases according to the technique of Bligh and Dyer (1956). To ensure complete separation, the test tubes were centrifuged using a bench top centrifuge for 5 minutes at 2200 rpm.

SEPARATION AND QUANTIFICATION OF REACTION PRODUCTS OF THE PLD ASSAY

Phosphatidylcholine is hydrolyzed by PLD in a reaction that gives rise to phosphatidate and water soluble choline. Most of the previous studies on PLD usually involved labelling of PC with [^{3}H]myristate. A primary alcohol was added which served as the acceptor for the phosphatidyl moiety in a transphosphatidylation reaction catalyzed by PLD. The metabolically stable [^{3}H]phosphatidyl alcohol was then separated from the rest of the phospholipids in the cellular extracts by tlc.

The method employed in this thesis involved labelling of PC with [^{3}H]choline. After the reaction, water-soluble products are isolated. Separation of [^{3}H]choline from [^{3}H]choline phosphate is achieved by passing the water soluble products through a column containing Dowex, a cation exchange resin (Cook and Wakelam, 1989). The idea behind this separation is that by isolating [^{3}H] choline from [^{3}H] choline phosphate one can distinguish between the activities of PLD and PLC, whose products would be choline and choline phosphate, respectively. Samples (700 µl) of the top phase of the Bligh-Dyer extraction were added to a syringe barrel filled with 10 ml of nanopure water. The smaller end of the syringe was fitted with a Pasteur pipet that was plugged with glass fiber and approximately 1 inch of a 50 % slurry of Dowex 50X8 200 mesh. The sample was allowed to pass through the Dowex column. During this loading of the sample to the resin, choline would bind very strongly whereas most of choline phosphate would pass through and be found in the eluate. The column was washed with another 10 ml of nanopure water and the bound [^{3}H]choline was eluted with the addition of 10 ml of 1 M KCl. This fraction was collected directly in a scintillation vial and 10 ml of scintillation fluor was added. The radioactivity was detected by scintillation counting.

In order to characterize the separation of choline from choline phosphate and to prove that this separation technique provides accurate measurements of [^{3}H]choline, individual columns were prepared using the method described above. Two different mesh sizes of Dowex were used. Dowex 50X8-400 was the resin originally used by Cook and Wakelam (1989), whereas Dowex 50X8-200 was used in the present studies because of its larger particle size. This decreases the time required for the solvent to pass through the column by a factor of ten. An aqueous solution containing approximately 100,000 dpm of [^{3}H]choline and 100,000 dpm [^{14}C]choline phosphate was added to each column filled with 10 ml of nanopure water . The columns were allowed to drip and the eluant was collected. The columns were then washed five times with 2 ml of nanopure water. Lastly, the bound choline was eluted using 10 ml of a 1 M KCl solution in 2 ml aliquots. Ten ml of scintillation fluor was added to all eluted fractions that were collected. These were counted using a dual label program that measured the radioactivity of both ^{3}H and ^{14}C simultaneously. Figure 1 shows the elution profile obtained from this single experiment performed in duplicate. Both 400 and 200 mesh Dowex were very efficient in separating choline phospate from choline. The recovery of each labeled compound and cross-contamination of the samples were calculated. Only 2% of the choline phosphate counts were carried over to the fractions in which choline was eluted when the 400 Dowex was used; similarly, less than 7% of the same counts were carried over with the bigger 200 mesh Dowex. Furthermore, the mixed-labeled compound sample added was in a salt-free solution. In the actual reaction conditions used in the assay the methanolic aqueous phase that is subjected to this separation, and the presence of 400 mM salt will probably eliminate this slight stickiness of choline phosphate.

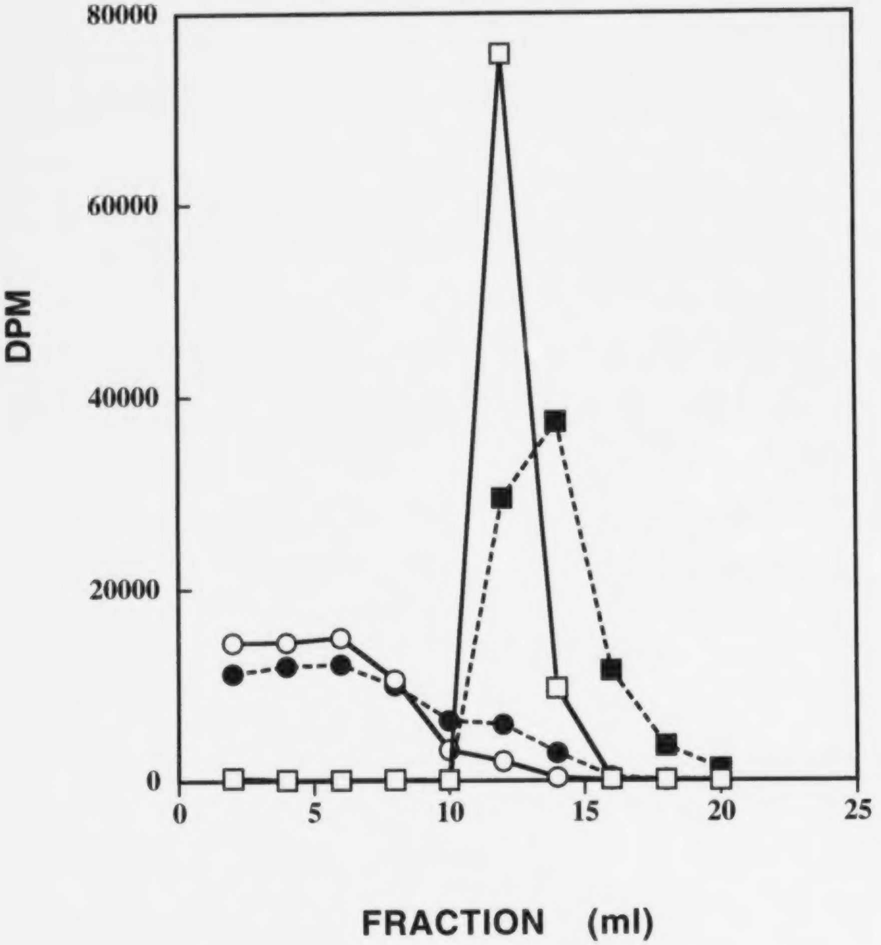
Overall, more than 96 % recovery of [^{3}H]choline was observed in the fraction (eluted with 10 ml KCl) that would normally be collected for scintillation counting, proving that this separation is very efficient and reliable and gives an accurate measure of PLD activity in this assay system.

PURIFICATION PROTOCOL FOR GLU-TAGGED ARF FROM BV Sf 9 CELLS

Cells were lysed in hypotonic lysis buffer (HLB) containing 20 mM Tris-HCl pH 8.3, 1 mM EGTA, 1mM $MgCl_2$, 1 mM DTT, 1mM pefabloc, 10 µq/ml aprotinin, 10 µg/ml leupeptin, 1% nonidet NP-40. The cell lysate was spun at 13000 rpm at 4 C for 20 min. The supernatant was removed and loaded onto an anti-Glu PGS column at 4 C.

FIG. 1 **THE ELUTION PROFILE OF CHOLINE AND CHOLINE PHOSPHATE BY ION-EXCHANGE CHROMATOGRAPHY USING DOWEX 50X8 COLUMNS.**

An aqueous sample containing a mixture of [Me-^{3}H]choline and [^{14}C]choline phosphate of known radioactivity was applied to a column containing either Dowex 400 (open symbols) or 200 (closed symbols). The columns were washed with 10 ml of nanopure water and 10 ml of 1 M KCl and the eluant was collected at 2 ml fractions. Open circles depict choline elution with Dowex 400, closed circles depict choline elution on with Dowex 200. Open squares depict choline phosphate elution with Dowex 400, closed squares depict choline phosphate elution with Dowex 200. Results are from a single experiment performed in duplicate.


80000
60000
40000
20000
0
DPM
0
5
10
15
20
25
FRACTION (ml)

The column was then washed 3 times with 10 column volumes of 1) HLB, 2) HLB without 1% NP-40, 3) HLB plus 400mM NaCl without 1% NP-40. The epitope tagged ARF was eluted from the column with a buffer containing HLB plus 400mM plus 100 µg/ml Glu-Glu peptide. The collected fractions were tested for purity by SDS-PAGE.

PREPARATION AND PURIFICATION OF GST-RhoA FUSION PROTEIN

E. coli transfected with pGEX-2T vector were grown in L broth for 1 hour prior to induction with 1 mM IPTG. Cells were pelleted and resuspended in buffer containing 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF. The sespension was sonicated, spun at 10000 rpm, and the supernatant was collected and added to glutathione-sepharose beads with swirling at 4 C for 30 min. The beads were then spun down and washed three times with cold lysis buffer. The beads were resuspended in thrombin resuspension buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM DTT) thromin (3 units per 2 ml) was added and the mixture was incubated overnight. The beads were spun down and the supernatant was collected. Thrombin was removed with 10 µl of p-aminobenzamidine-agarose beads. The beads were spun and the supernatant was collected, concentrated and checked for purity with SDS-PAGE.

SDS-PAGE GELS

The SDS-PAGE gels were prepared using the BIO-RAD mini-gel apparatus according to instruction notes from the company. The gels were run vertically using a BIORAD power supply. The composition of the gels were those previously reported by

Laemmli (1970). In more detail, the composition of the 3% stacking gel and the 10 or 12% resolving gel was as follows:

3% stacking gel:

volume (ml)	component
6.35	distilled water
2.50	0.5 M Tris/HCl, pH 6.8
1.00	30% (w/v) acrylamide, 1% bis-acrylamide
0.10	10% SDS
0.05	10% (w/v) ammonium persulfate
0.01	N,N,N',N'-Tetramethylethylenediamine

10% resolving gel

volume (ml)	component
11.35	distilled water
5.00	1.5 M Tris/HCl, pH 8.8
3.40	30% (w/v) acrylamide, 1% bis-acrylamide
0.20	10% SDS
0.1	10% (w/v) ammonium persulfate
0.02	N,N,N',N'-Tetramethylethylenediamine

WESTERN BLOT ANALYSIS

Proteins were transferred from SDS-PAGE gels onto Immobilon P membranes for 2 hours at the highest voltage setting using a transfer apparatus. The immunoblots were blocked overnight with 1% (w/v) BSA and incubated with a primary monoclonal antibody (1:5000 dilution in blocking buffer) for either Rho A (a gift from Dr. Allan Hall, University

College, London), or ARF 1 (a gift from Dr. Simon Cook, Onyx Pharmaceuticals, California), for 2 hours. The blots were then incubated for 1 hour with a secondary antibody (anti-sheep IGg conjugated to horse radish peroxidase). Blots were developed using the ECL kit from Amersham according to the manufacturer's instructions.

RESULTS

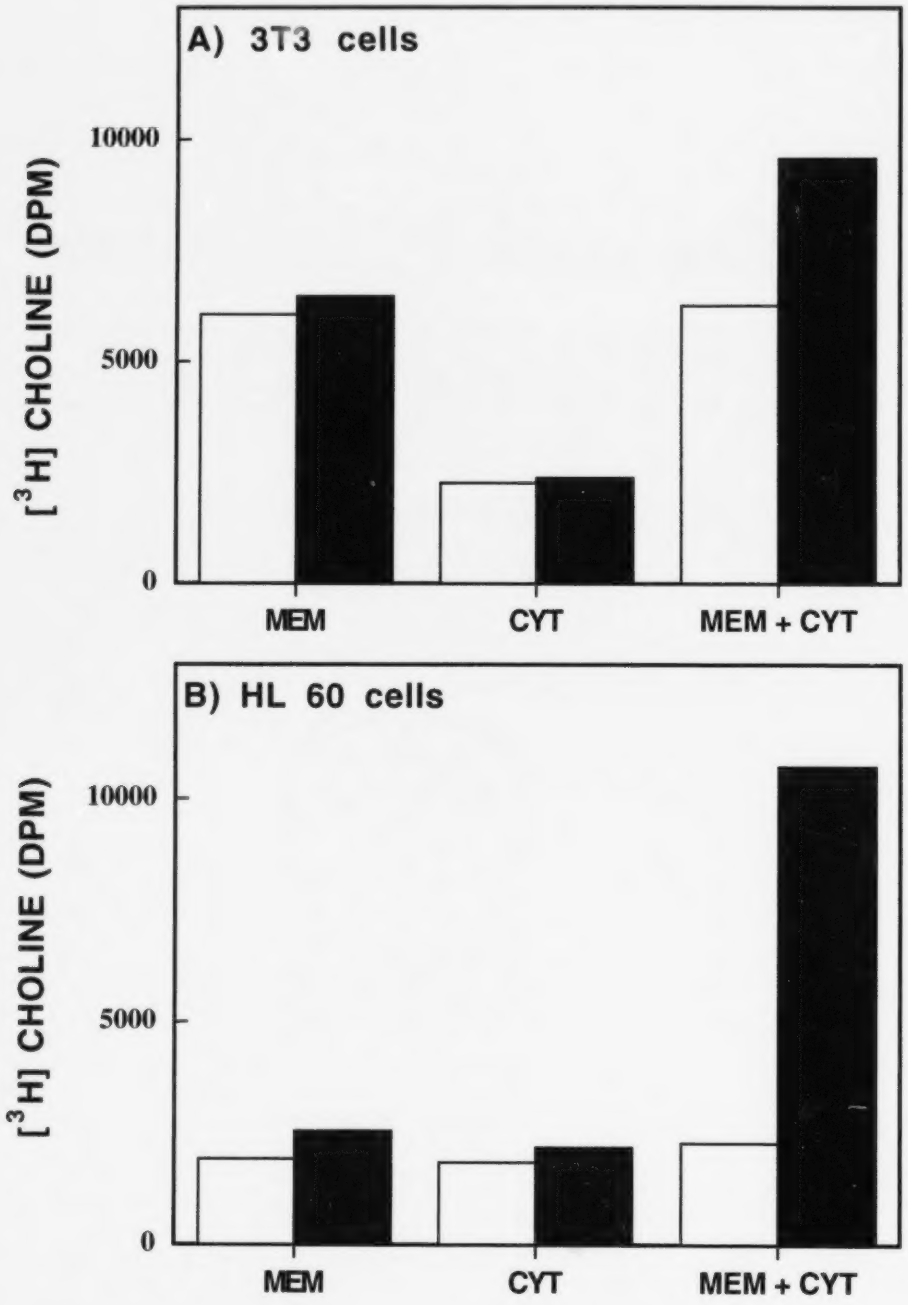
CHARACTERIZATION OF THE CELL-FREE PLD ASSAY IN SWISS 3T3 FIBROBLASTS AND HL 60 CELLS

Previous studies (Olson et. al. 1991, Anthes *et al.* 1991) have shown that guanosine 5'-O -(3-thiotriphosphate) (GTPγS) can activate PLD in a Ca^{2+} dependent manner in human neutrophils or Chinese hamster lung fibroblasts. Furthermore this activation, which has been observed in a cell free system, requires the presence of both membranes and cytosol, indicating that both fractions contain components essential for the activation of PLD. Brown *et al.* (1993) also have shown the strong dependency of PLD activation on GTP γ S and the presence of membranes plus cytosol in HL 60 cells. The same assay system with slight modifications has been used throughout these studies on PLD activity and has shown very high reproducibility and very low variance (± 200 dpm).

To establish this assay in our laboratory, and for the purposes of future studies on the enzyme, a survey of PLD activity in HL 60 and Swiss 3T3 cells was performed. Cells were harvested as described in Materials and Methods, sonicated, and the cytosol was separated from the membranes by high speed centrifugation. The cytosol, membranes, and the combination of the two were assayed for PLD activity by presenting to the enzyme a mixed phospholipid vesicle containing [^{3}H-methyl]PC, PIP_2, and PE. The reaction was performed as described in Materials and Methods. The results are summarized in Figure 2. Panel A depicts results obtained from Swiss 3T3 cells. More PLD activity was detected in 3T3 membranes than in cytosol (white bars) and this activity was not increased in the presence of 30 μM GTP γ S (black bars).

FIG. 2 **RECONSTITUTION OF PLD ACTIVITY *IN VITRO* IN SWISS 3T3 AND HL 60 CELLS.**

Confluent 3T3 and HL 60 cells were harvested according to the procedure described in Materials and Methods. Membranes, cytosol, or membranes plus cytosol were assayed according to the *in vitro* PLD assay described in Materials and Methods. Panel A depicts results obtained from Swiss 3T3 cells ; panel B represents results obtained from HL 60 cells. Open bars depict results were GTP γ S was omitted. Closed bars depict results were 30 μM GTP γ S was added. Results are representative of one of two identical independent experiments.


A) 3T3 cells
[^{3}H] CHOLINE (DPM)
10000
5000
0
MEM
CYT
MEM + CYT
B) HL 60 cells
[^{3}H] CHOLINE (DPM)
10000
5000
0
MEM
CYT
MEM + CYT

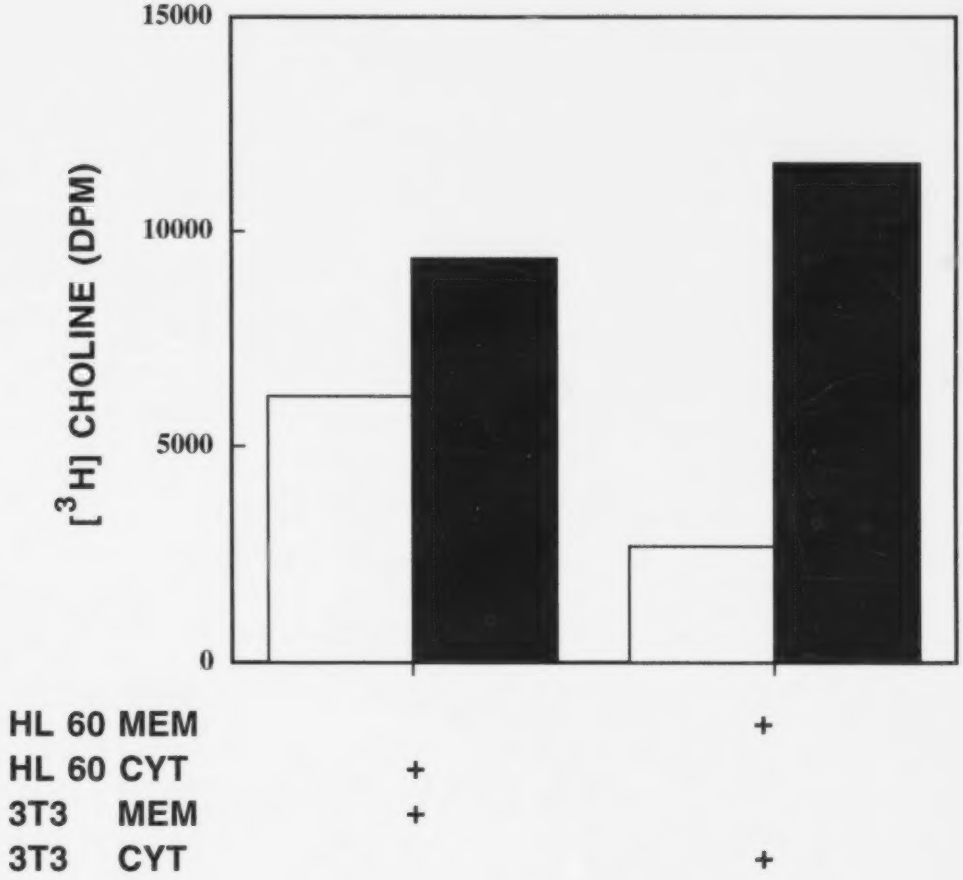
A 0.5 fold activation was observed when cytosol and membranes were mixed together in the presence of 30 μM GTPγS, indicating the additive effect of membranes plus cytosol in activating PLD. In panel B, where the results from HL 60 cells are shown, the same pattern of activity profile was observed. There was little or no detectable activity in membranes or cytosol alone in the presence or absence of GTP γ S (white and black bars, respectively), but the combination of the two resulted in a 4-fold activation of PLD in the presence of GTP γ S. This compound is a non-hydrolyzable analog of GTP which cannot be metabolized by GTPases. When bound to G proteins, GTP γ S renders them constitutively active. The activation observed in the presence of this nucleotide in both cell lines suggests the involvement of G protein(s) in the activation of PLD. Both membranes and cytosol are required for this activation, especially for HL 60 cells.

Using streptolysin O to permeabilize cells, Cockcroft (1992) further showed that the factor involved in the activation of PLD is cytosolic. In the experiment illustrated in Figure 3, cytosol from HL 60 cells was mixed with membranes from 3T3 and the activity of PLD was measured in the presence or absence of GTPγS. The results show that the HL 60 cytosol can substitute for the 3T3 cytosol. The same also holds true for the reciprocal experiment. This indicates that the G binding factor(s) is/are expressed in different cell types and its ability to activate PLD is not a cell type specific phenomenon.

Overall, the results obtained here are in accord with those obtained by Cockcroft *et al.* (1994) and Brown *et al.* (1993) in that GTP γ S can stimulate the activity of PLD in a cell free system, and, in order to reconstitute this activity, both membranes and cytosol are required. In the case of Swiss 3T3 cells, the same activity profile as in HL 60 cells was observed, although the relative activation observed was lower than that observed in the HL 60 cell line. This observation could be due to PLD being unavailable for further stimulation, since considerable membrane activity was detected in the absence of GTPγS.

FIG. 3 **CYTOSOL FROM HL 60 CELLS CAN ACTIVATE MEMBRANE-BOUND PLD FROM SWISS 3T3 CELLS AND VICE VERSA.**

The first two columns represent the results obtained from the addition of HL 60 cytosol to Swiss 3T3 membranes and the last two represent the results obtained when 3T3 cytosol was added to HL 60 membranes. Both membranes and cytosol, from either cell line, were present at the same protein concentration (20 μg). The mixture of the two components was assayed according to the method described in Materials and Methods section. Open bars depict results where GTPγS was omitted. Closed bars depict results where 30 μM GTPγS was added. Results are representative from one of two experiments performed in duplicate.


15000
10000
5000
0
[3H] CHOLINE (DPM)
HL 60 MEM +
HL 60 CYT +
3T3 MEM +
3T3 CYT +

THE EFFECTS OF VANADATE, N-ETHYLMALEIMIDE, METAL IONS, GDPβS, AND THE ARF N-TERMINAL PEPTIDE ON THE RECONSTITUTED PLD ACTIVITY IN VARIOUS CELL LINES

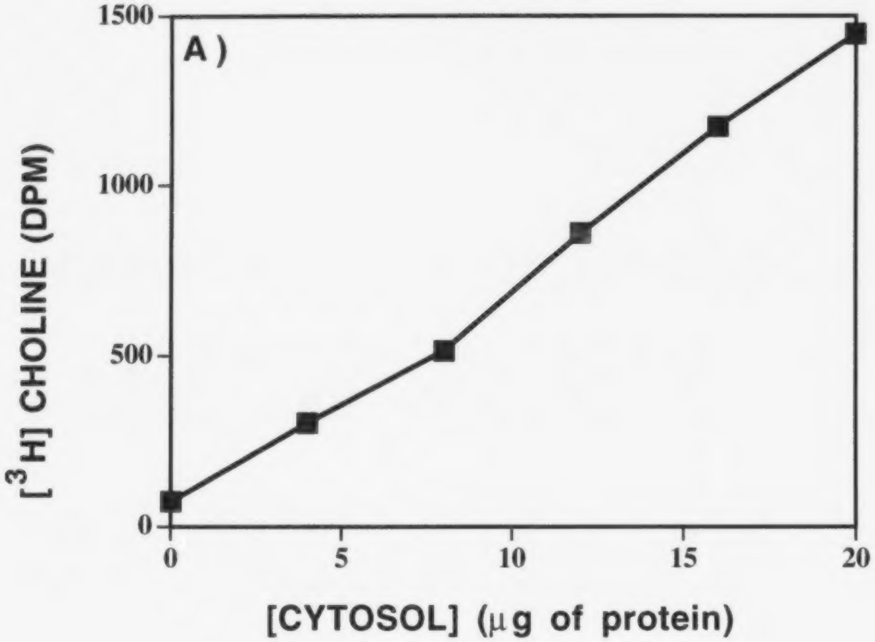
To characterize further the reconstituted PLD activity observed in the previous section, an additional series of experiments were performed in Swiss 3T3, HL 60 and Rat 1 cell lines. As was shown in Figure 2 panel B, addition of HL 60 cytosol to HL 60 membranes in the presence of GTPγS resulted in a 3.5 fold activation of PLD. Figure 4 panel A illustrates that the PLD activation, observed upon addition of cytosol, was concentration dependent and was still sub-maximal when 20 µg of cytosol protein were added. For the purposes of the experiments presented in this thesis (keeping the reaction volume at 100 µl), 20 µg of cytosolic protein was found to be sufficient for activation of PLD and was therefore used in every assay (unless otherwise stated).

Bourgoin and Grinstein (1992) showed that peroxides of vanadate can lead to the activation of PLD in HL 60 cells. Vanadate is a potent tyrosine phosphate phosphatase inhibitor and its presence can lead to an accumulation of tyrosine phosphorylated proteins. To examine the effect of this inhibitor in the reconstituted PLD assay increasing amounts of sodium vanadate were added to enzyme reactions containing membranes plus cytosol, and GTP γ S. The results are summarized in Figure 4 panel B. Vanadate (0.1 mM) inhibited the PLD activity observed in HL 60 cells by more than 50 % and at 1 mM the activity was almost completely abolished.

The PLD activity in Swiss 3T3 cells was also completely inhibited in the presence of 5 mM N-ethylmaleimide, which acts as a sulfhydryl alkylating agent (Figure 5). Brown *et al.* (1993) have shown that 50 % of the PLD activity found in HL 60 membranes can be extracted using NaCl at 400 mM. No significant difference compared to the controls

FIG 4 CONCENTRATION CURVES OF CYTOSOL AND VANADATE VERSUS HL 60 MEMBRANES.

HL 60 membranes were prepared according to the appropriate section in Materials and Methods. HL 60 membranes (10 μg of protein) were used for each point along with 30 μM GTPγS. Panel A depicts the effect of increasing concentration of cytosolic protein on the membrane bound PLD. Panel B shows the effect of increasing concentration of sodium vanadate on the PLD activity of HL 60 membranes plus HL 60 cytosol in the presence of 30 μM GTPγS after a 90 minute incubation at 37°C. Results shown are from a single experiment performed in duplicate.


A)
[³H] CHOLINE (DPM)
1500
1000
500
0
0
5
10
15
20
[CYTOSOL] (μg of protein)

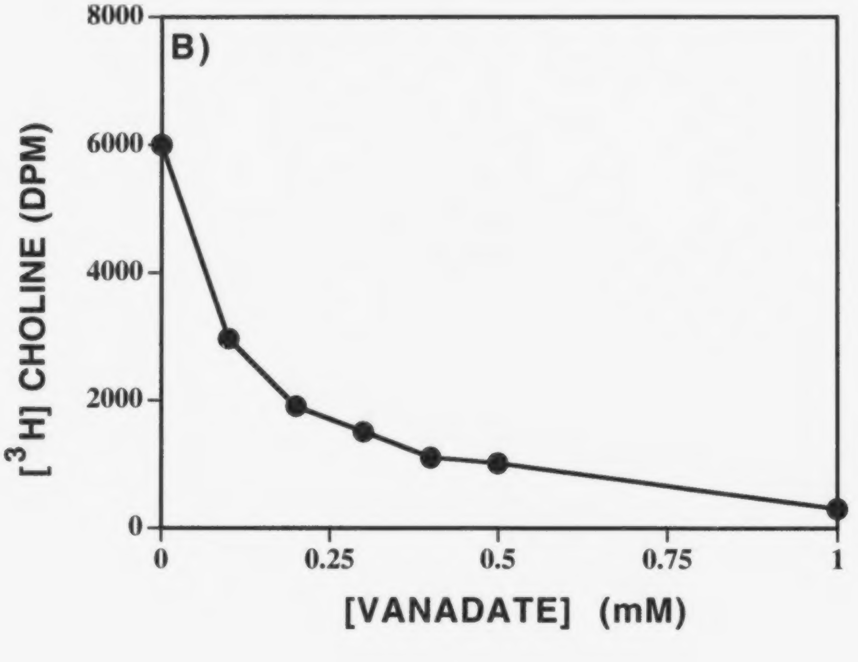
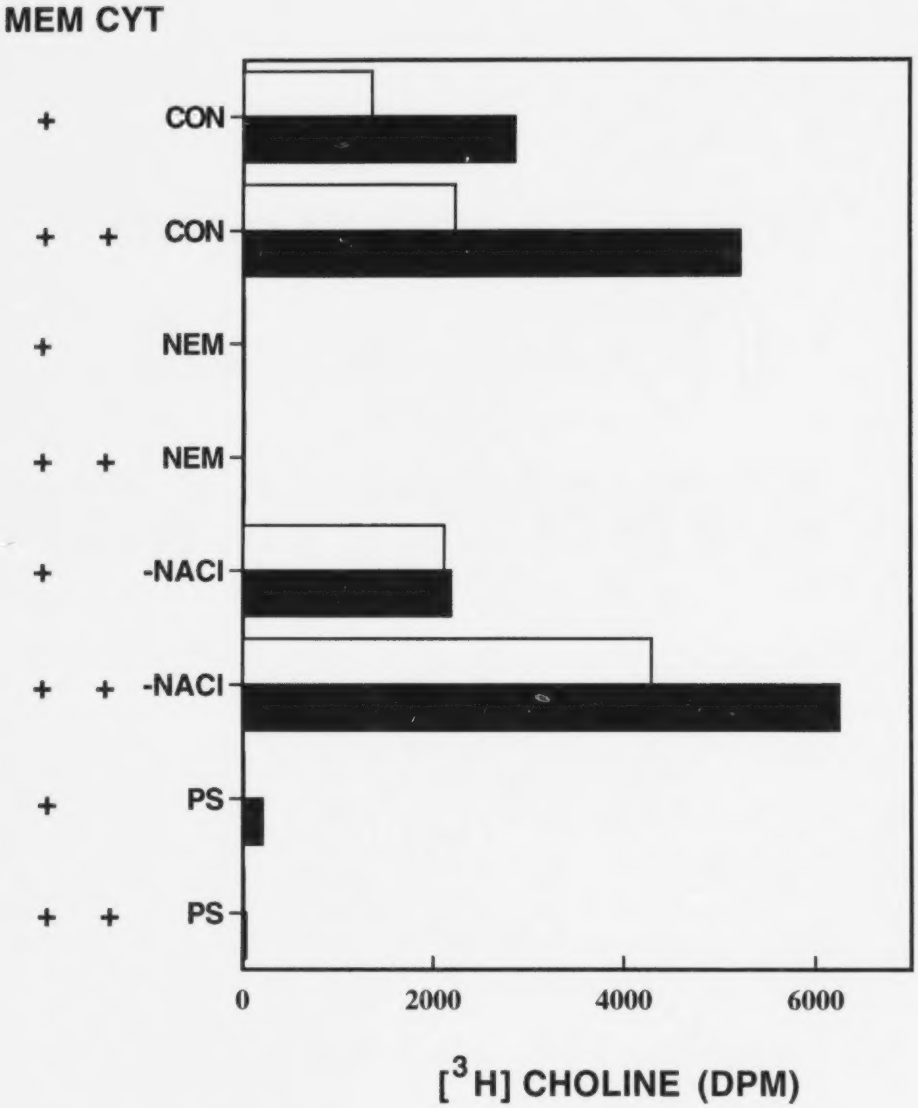

B)
[³H] CHOLINE (DPM)
8000
6000
4000
2000
0
0
0.25
0.5
0.75
1
[VANADATE] (mM)

FIG. 5 **THE EFFECT OF, THE SULPHYDRYL REAGENT NEM, NaCl, AND PHOSPHATIDYLSERINE ON THE PLD ACTIVITY OF SWISS 3T3 MEMBRANES AND MEMBRANES PLUS CYTOSOL.**

Swiss 3T3 membranes and cytosol were prepared as described in the Materials and Methods section. The graph depicts the results when different conditions, i.e. the presence of 5 mM N-ethyl maleimide (NEM), or the absence of 400 mM NaCl (- NaCl), or the substitution of 12 μM phosphatidyl inositol-4,5 bisphosphate (PIP_2) from the substrate micelle with 12 μM phosphatidylserine (PS), were assayed in the presence (black bars) or absence (white bars) of GTPγS. All assays contained 20 μg of cytosolic and 20μg of membrane protein. Results are representative from one of two experiments performed in duplicate.


MEM CYT
+ CON
+ + CON
+ NEM
+ + NEM
+ -NACl
+ + -NACl
+ PS
+ + PS
0
2000
4000
6000
[^{3}H] CHOLINE (DPM)

(i.e., membranes or membranes plus cytosol in the presence of GTPγS) was observed when 400 mM NaCl, which is normally present in the reaction mixture, was omitted from the assays containing either Swiss 3T3 cytosol and membranes (Figure 5) or cytosol and membranes from Rat 1 fibroblasts (Figure 7, top panel). A slight activation of cytosolic PLD (3T3 cells) in the presence of GTPγS was observed under the same conditions . Furthermore, omission of 3 mM Ca^{2+} and 2 mM Mg^{2+} , which are normally used in the assay, had no effect on the PLD activity in Swiss 3T3 cells (Figure 6), Rat 1 fibroblasts (Figure 7), or HL 60 cells (Martin, personal communication), although Geny *et al.* (1992) have reported an increase in PLD activity upon addition of 10 μM Ca^{++} in permeabilized HL 60 cells..

Being a non-hydrolyzable non-phosphorylatable form of GDP, GDPβS will be bound by G proteins and render them inactive. The presence of 3 mM GDP β S resulted in more than a 50 % reduction in the reconstituted PLD activity that is stimulated by 30 μM GTP γ S (Figure 6 for Swiss 3T3, and Figure 7 for Rat 1 fibroblasts). This finding further suggests the involvement of a G protein in activating PLD. Brown *et al.* (1993) and Cockcroft (1994) have shown that the G protein involved in the activation of PLD is a 21 kDa protein known as ADP ribosylation factor, ARF. The GTPγS dependent activity observed in Swiss 3T3 cells was completely inhibited when an N-terminal peptide of ARF was included in the assay (Figure 6). This provided further proof that the GTP γS stimulation of PLD was mediated by ARF.

Liscovitch *et al.* (1994) have reported that PIP_2 is a potent activator of PLD with an EC_{50} of less than 1 mol %. In addition, Brown *et al.* (1993) have also shown the absolute requirement of PIP_2 in the substrate liposome when assaying PLD activity in HL 60 cells. In the present work, the *in vitro* PLD activity of Swiss 3T3 cells was absolutely dependent on the presence of 12 μM PIP_2 in the substrate liposome because substitution of the latter with PS at the same concentration, resulted in a complete loss of PLD activity (Figure 5).

FIG. 6 **THE EFFECT OF, METAL IONS, GDPβ S, AND THE N-TERMINAL ARF PEPTIDE INHIBITOR, ON THE PLD ACTIVITY OF SWISS 3T3 MEMBRANES, AND MEMBRANES PLUS CYTOSOL.**

Swiss 3T3 cytosol and membranes were prepared and assayed for PLD activity as described in the Materials and Methods. The graph shows the effects of omitting Ca^{++} or Mg^{++} from the reaction mixture, adding 3 mM GDPβS, or adding the N-terminal ARF peptide (1 μg of protein) to assays containing membranes or cytosol plus membranes (all additions at 20 μg of protein each) in the presence (black bars) or absence (white bars) of 30 μM GTPγS. Results are representative from one of two experiments performed in duplicate.

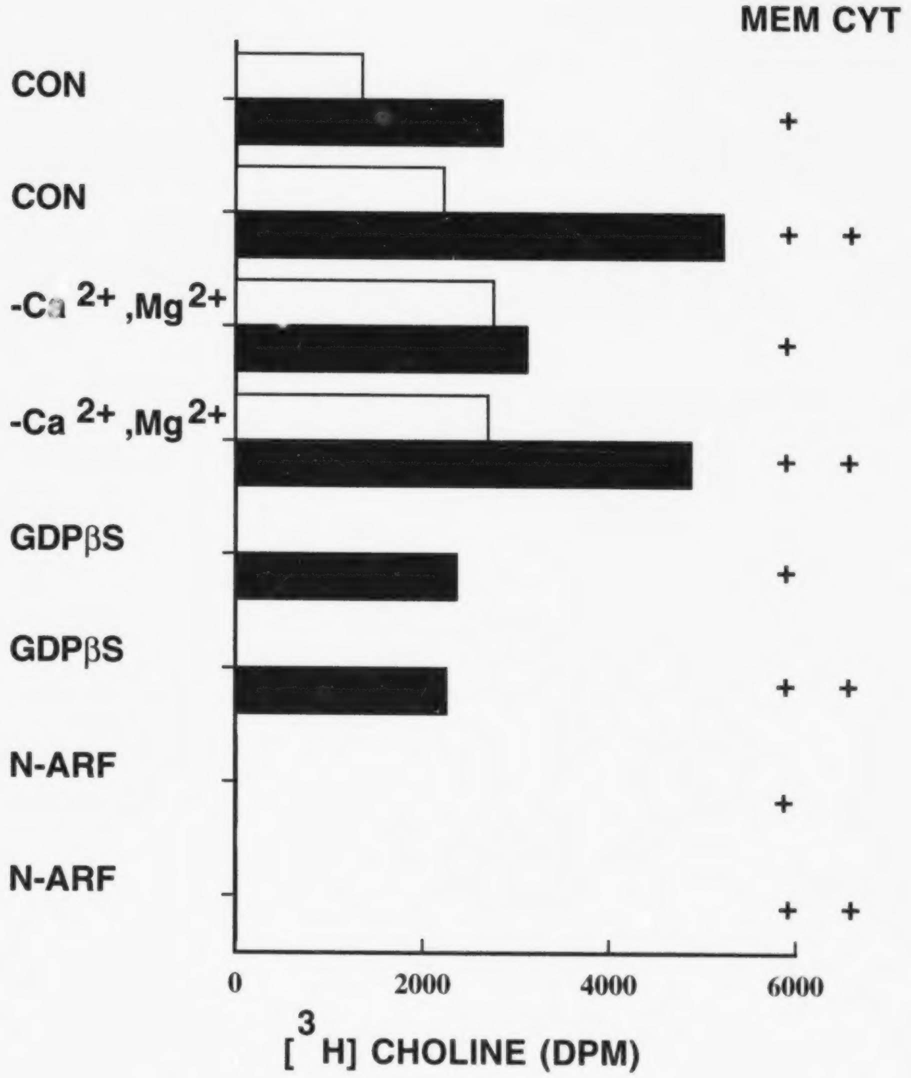
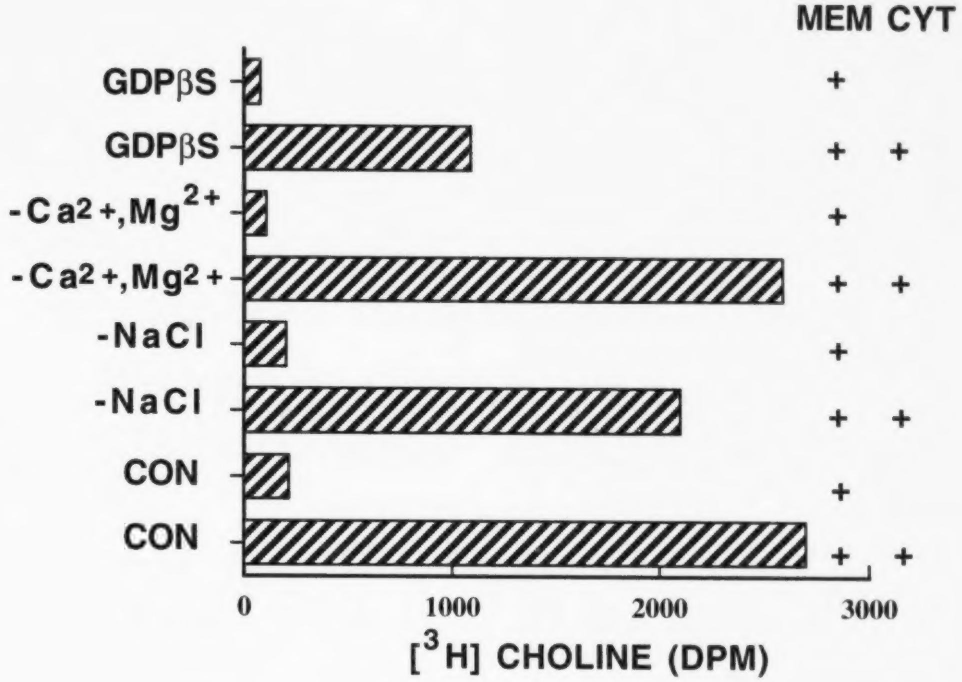
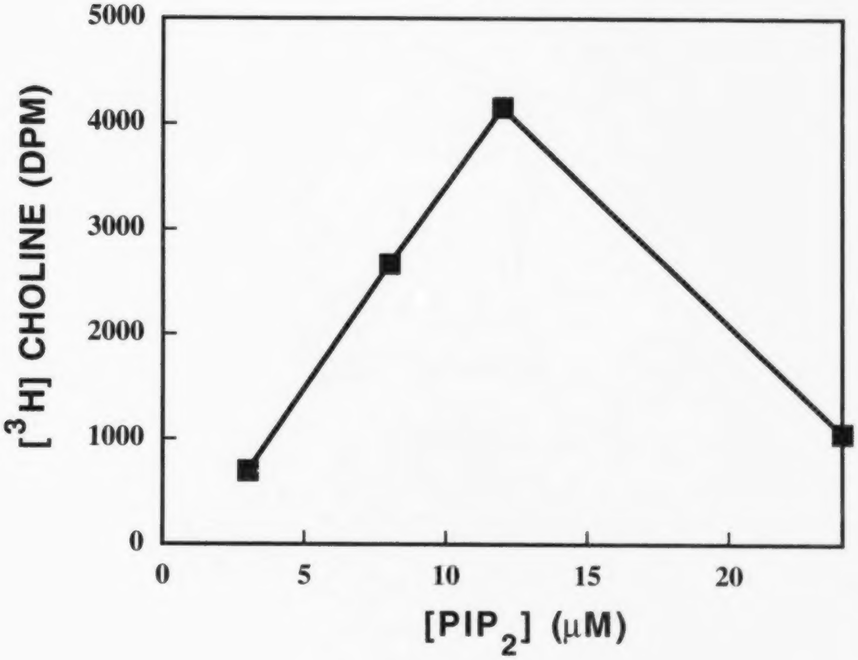

MEM CYT
CON
+
CON
+ +
-Ca 2+ ,Mg 2+
+
-Ca 2+ ,Mg 2+
+ +
GDPβS
+
GDPβS
+ +
N-ARF
+
N-ARF
+ +
0
2000
4000
6000
[3H] CHOLINE (DPM)

FIG. 7 EFFECT OF METAL IONS, GDPβS, NaCl, AND PHOSPHATIDYL INOSITOL-4,5 BISPHOSPHATE ON THE PLD ACTIVITY OF RAT 1 FIBROBLASTS.

Rat 1 fibroblasts were collected and treated essentially the same way as Swiss 3T3 cells as described in the relevant section in Materials and Methods. The top panel represents results obtained when Ca^{++}, and Mg^{++} or NaCl was omitted from the reaction mixture, and GDPβS was included in assays containing cytosol or membranes plus cytosol in the presence of GTPγS (hatched bars). The bottom panel summarizes the activity profile obtained when membranes plus cytosol were assayed in the presence of increasing concentrations of phosphatidyl inositol-4,5 bisphosphate (PIP_2). Results for the top panel are representative of one of two experiments performed in duplicate and the results from the bottom panel are from one experiment performed in duplicate.


MEM CYT
GDPβS +
GDPβS + +
-Ca2+,Mg2+ +
-Ca2+,Mg2+ + +
-NaCl +
-NaCl + +
CON +
CON + +
0
1000
2000
3000
[3H] CHOLINE (DPM)


5000
4000
3000
2000
1000
0
[3H] CHOLINE (DPM)
0
5
10
15
20
[PIP2] (μM)

The stimulatory effect of PIP_2 seems not to be due solely to its negative charge since the substitution with another negatively charged phospholipid (PS) did not produce the same results. The stimulatory effect of PIP_2 was also observed in Rat 1 fibroblasts where a concentration curve was performed. The PIP_2 concentration required for optimal activation of PLD activity in Rat 1 fibroblasts was 12 μM; whereas, 24 μM PIP_2 was inhibitory probably due to disruption of the substrate liposome (Figure 7, bottom panel).

In summary, in HL 60 cells the cytosol-dependent activation of membrane PLD, in the presence of GTPγS, was concentration-dependent. The enzyme activity was NEM-sensitive (3T3 cells) and was completely inhibited by sodium vanadate (HL 60 cells). Neither metal ion (3 mM Ca^{2+} and 2 mM Mg^{2+}) nor salt was required for the activation of PLD in Swiss 3T3 and Rat 1 fibroblasts. The N-terminal peptide of ARF completely inhibited the GTPγS dependent activity of PLD (3T3 cells). Kahn *et al.* (1992) has shown that the N-terminal part of ARF is involved in associating with other target and downstream proteins and is considered to be the “business end” of the molecule. Taken together with the inhibition observed when 3 mM GDPβS was added, these results provide strong evidence for the characterization of the involvement of ARF in activating PLD. The PLD activity also has an absolute requirement for PIP_2 which at 12 μM in the substrate liposome resulted in activation of PLD (rat-1 cells); however, a higher concentration of PIP_2 (12 μM) was inhibitory. The implications of this PIP_2 requirement that has been observed in a variety of cell lines will be discussed in Chapter 3 (Discussion and further experiments).

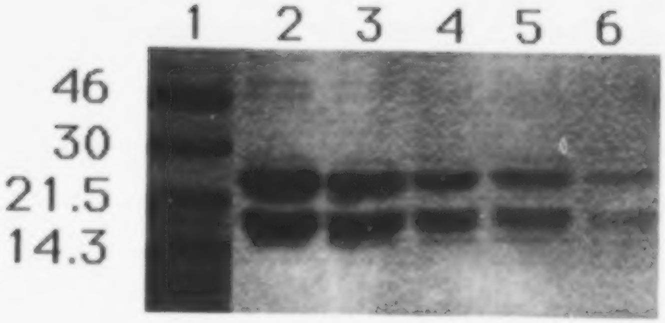
PURIFICATION OF ADP RIBOSYLATION FACTOR (ARF) FROM INSECT SF 9 PELLETS AND CHARACTERIZATION OF ARF REGULATED PLD ACTIVITY

Two independent publications (Brown *et al.*, 1993, Cockcroft *et al.*, 1994) have reported the purification of a cytosolic component that confers sensitivity to GTPγS for PLD activity. This factor was identified as ARF. This conclusion was confirmed with the purification of recombinant ARF proteins using a reconstituted assay system. Furthermore, Brown *et al.* (1993) provided evidence suggesting that partial myristoylation of ARF results in a protein that is a better activator of PLD than the non-myristoylated form. Franco *et al.* (1995) showed that N-myristoylation of ARF facilitates nucleotide exchange at high Mg^{2+} concentrations (mM range) by allowing GDP dissociation in the presence of phospholipids. Therefore the subsequent binding of GTP to this form renders the myristoylated form more active than its non-modified counterpart.

For the purposes of our studies we also purified recombinant ARF from insect SF 9 cells that had been transfected with a vector containing the full length ARF 1 sequence using a standard protocol involving affinity chromatography of epitope tagged (Glu-Glu) proteins as described in the Materials and Methods section. The selection of insect cells as the host cell of the vector was chosen over bacteria because the former are known to contain the biochemical machinery necessary for N-myristoylation of proteins containing signal sequences that would trigger co-translational myristolylation of N-terminal glycine. Figure 8 shows the results from the concentrated peak fractions collected from the anti-Glu PGS column on 12 % SDS-PAGE. After Coomassie blue staining, two major doublets were detected with an apparent electrophoretic mobility of about 19 and 23 kDa. The presence of other contaminants was minor and the purity of the preparation was estimated to be > 95%.

FIG. 8 **PURIFICATION OF EPITOPE TAGGED Glu-Glu ARF1 AND SEPARATION OF MYRISTOYLATED AND NON-MYRISTOYLATED FORMS OF ARF BY GEL FILTRATION.**

Upper gel: decreasing concentrations of ARF, which was purified according to the method outlined in Materials and Methods, were subjected to SDS-PAGE in a 12% gel and stained with Coomassie Blue. *Lane* 1, molecular mass markers (kDa); *Lanes* 2-6 7.5, 1.5, 0.75, 0.5, and 0.375 of µg of purified protein added. *Lower gel* : compared electrophoretic mobilities of the three forms of ARF after gel matrix chromatography . *Lane* 1, molecular mass markers (kDa); *Lane* 2, purified ARF before chromatography; *Lane* 3, sample from first major protein peak eluted from gel filtration chromatography (subsequently named band 1); *Lane* 4, sample from second major protein peak eluted from gel filtration chromatography (subsequently named band 2).


1
2
3
4
5
6
46
30
21.5
14.3

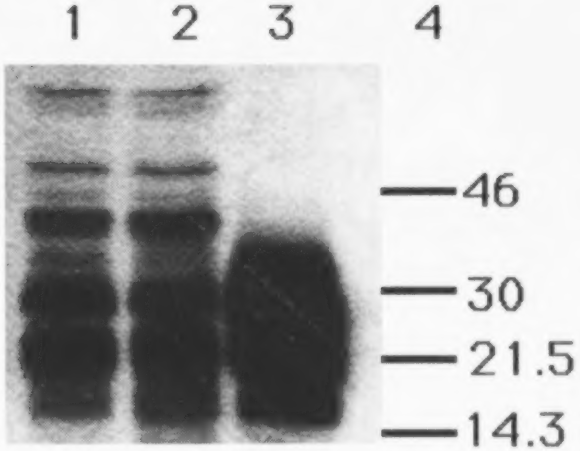
1
2
3
4
46
30
21.5
14.3

To show further that the purified product was indeed ARF, we performed a Western blot analysis using a monoclonal antibody against ARF, the resulting cross-reactivity was visualized using a secondary antibody coupled to horseradish peroxidase. Lane 3 from Figure 9 represents a sample taken from the purified product. There is a strong cross-reactivity of the monoclonal antibody with two bands of apparent molecular weight of 19 and 23 kDa and no other major contaminants or cross reactive bands are present. Compared to the bands obtained in lanes 1 and 2 which represent the supernatant from resuspended SF 9 cell pellets and the flow-through from the anti-Glu PGS column respectively, a small amount of the epitope tagged ARF was lost during the purification process. The appearance of cross reactive bands in lanes 1 and 2 are not usually seen in Western blots and are probably due to non adequate blocking or to cross reactivity of the secondary antibody with the transferred proteins. The resulting purified product contained mainly the two forms of ARF, i.e. myristoylated and non-myristoylated. The presence of a contaminating band of higher electrophoretic mobility than the myristoylated ARF was attributed to breakdown products. The appearance of two bands indicates the presence of both myristoylated and non-myristoylated forms of ARF. In addition, the recombinant ARF preparation contains approximately 50% from each of the myristoylated and non-myristoylated forms. Franco *et al.* (1995) reported that the myristoylated form of ARF has a greater electrophoretic mobility than does the non-myristoylated form. The extent of myristoylation of ARF in the Sf 9 expression system has been calculated to be about 50% (Cook S. , personal communication). Both of these observations are according to the results obtained here.

To determine the ability of the purified product to activate PLD, we assayed PLD activity in HL 60 membranes using increasing concentrations of ARF in the presence of GTPγS. The resulting concentration curve is shown in Figure 10. The stimulating activity of purified ARF on PLD was concentration dependent and not saturable at 25 nM of purified protein, which was essentially the yield of protein obtained from the Sf 9 cell pellets. This amount of ARF added to the assay does not indicate the exact amount of ARF required for GTP-dependent activation of PLD because not all the recombinant ARF was myristoylated (Figure 8).

FIG. 9 WESTERN BLOT ANALYSIS OF THREE MAJOR STEPS IN THE PURIFICATION PROTOCOL OF EPITOPE-TAGGED ARF FROM Sf-9 CELLS.

Samples from three different steps of the purification process of epitope tagged ARF. The cross reactivity of the monoclonal antibody against ARF (a gift from Dr. Cook, Onyx Pharmaceuticals, California) with proteins transferred onto nitrocellulose membrane was visualized using a secondary antibody coupled to horseradish peroxidase by ECL. *Lane* 1, sample from the supernatant obtained when resuspended SF 9 cell pellets were subjected to centrifugation according to the first step of the purification protocol; *Lane* 2, sample from the flow through from the anti-Glu PGS column; *Lane* 3, sample from the concentrated fractions eluted from the anti-Glu PGS column using a Glu-Glu peptide; *Lane* 4, molecular mass markers.


1
2
3
4
46
30
21.5
14.3

As it was pointed out earlier these two forms of ARF have different biochemical properties and do not activate PLD equally well. In order to prove that the myristoylated form of ARF is a better activator of PLD we employed a gel filtration chromatography technique to separate the two major bands obtained from the purified preparation of ARF (Figure 8, lower gel). The band with the higher electrophoretic mobility in a SDS-PAGE gel, or band 2, according to Franco *et al.* (1995) is ARF that has been myristoylated. Band 1 or the band that exhibits lower electrophoretic mobility, represents the portion of ARF that exists in the non-myristoylated form. In the presence of GTPγS, the myristoylated ARF, or band 2, is by far a better activator of PLD in membranes from either HL 60, or 3T3 cells than non-myristoylated ARF (Figure 11, panels A and B). In addition, the presence of recombinant ARF can substitute for the observed activation of PLD in the presence of cytosol, suggesting that the cytosolic component involved in such activation includes ARF.

Overall, we were able to isolate the epitope tagged recombinant ARF 1 from SF 9 cell pellets. The protein seems to be about 50 % myristoylated and is able to confer its effect on activating PLD, in a GTP γS dependent fashion, in membrane preparations from both HL 60 and Swiss 3T3 cells. The mere presence of this recombinant protein was sufficient for substituting the cytosolic requirement for activating membrane PLD.

FIG. 10 EFFECT OF PURIFIED EPITOPE TAGGED Glu-Glu ARF ON PLD ACTIVITY IN HL 60 MEMBRANES.

Recombinant ARF was purified according to the procedure outlined in the Materials and Methods section and was assayed, in the presence of 30μM GTPγS, for its ability to activate PLD in HL 60 membranes. The graph represents the $\log_{10}$ of ARF concentration versus the release of $[H^3]$choline in dpm . The results shown are from a single experiment performed in duplicate.

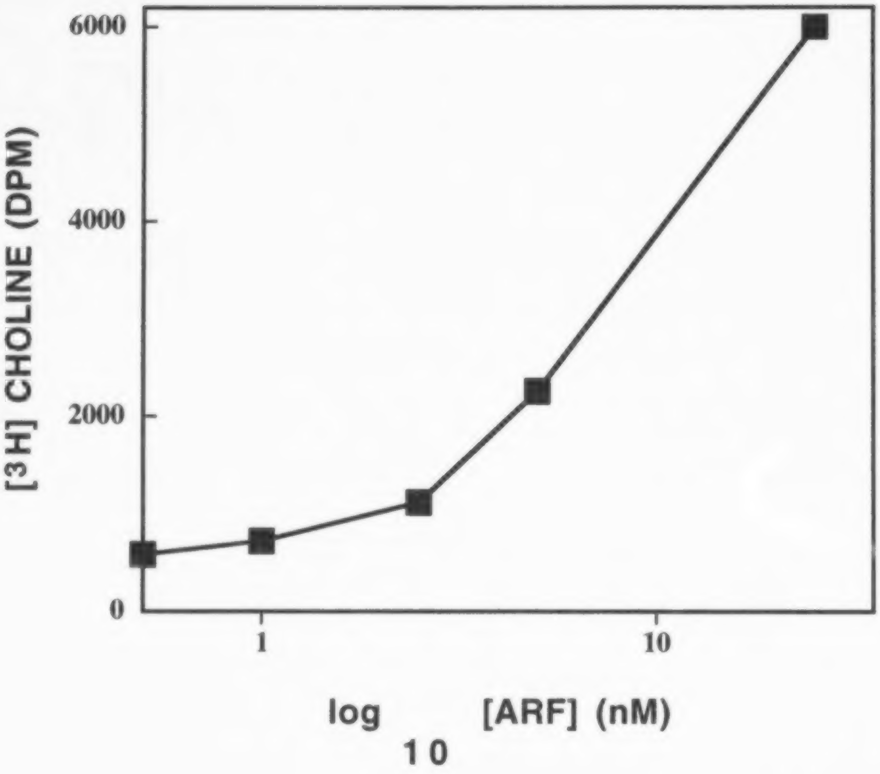
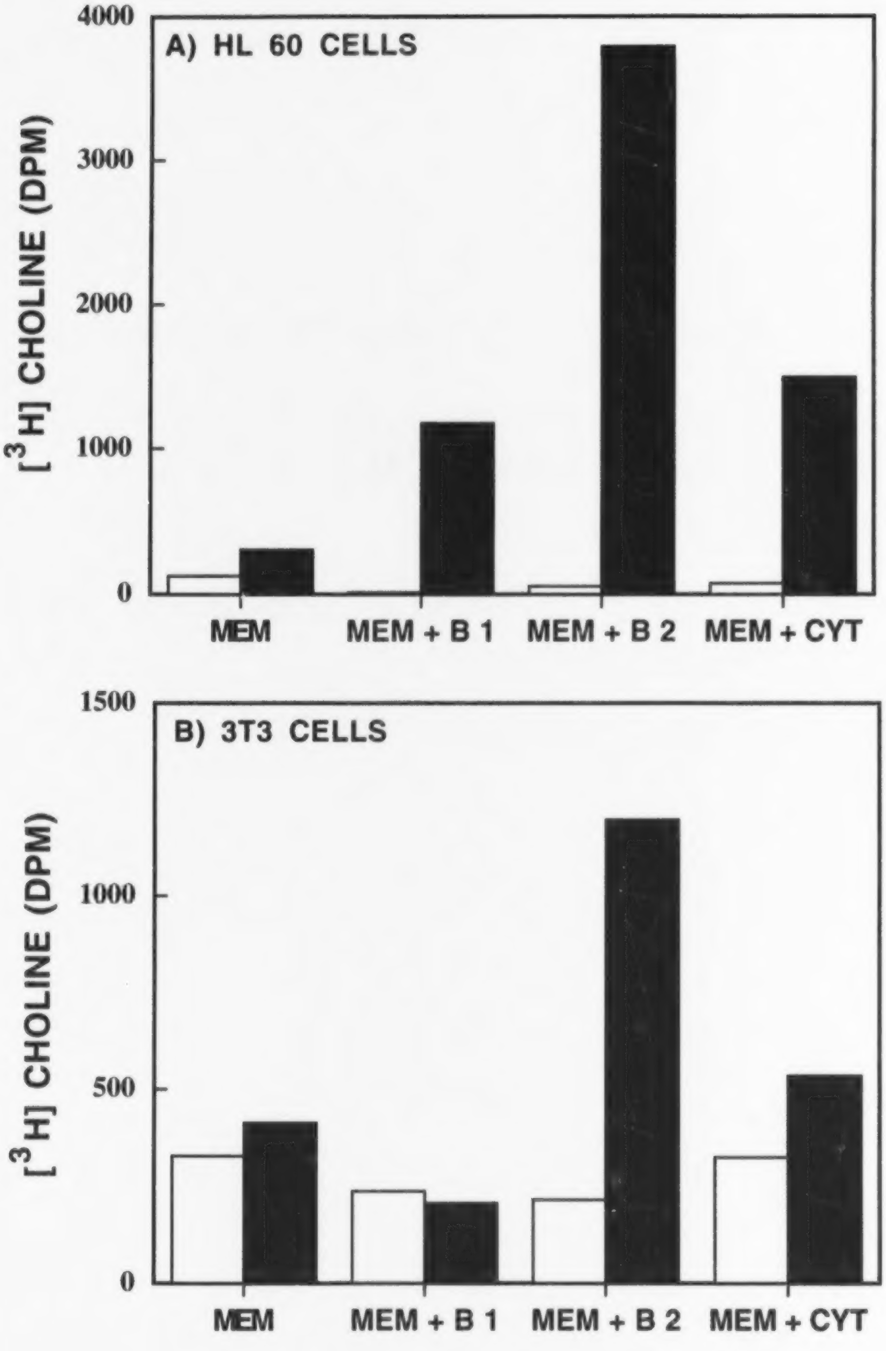

[3H] CHOLINE (DPM)
6000
4000
2000
0
1
10
log 10 [ARF] (nM)

FIG. 11 COMPARISON OF THE EFFECTIVENESS OF NON-MYRISTOYLATED ARF VERSUS MYRISTOYLATED ARF TO ACTIVATE PLD IN EITHER HL60, OR 3T3 MEMBRANES.

The two different forms of ARF were obtained using gel filtration chromatography as outlined in the Materials and Methods section. The figure represents results obtained when purified non myristoylated (B1) or myristoylated (B2) were incubated, at the same protein concentration, in the presence of HL 60 membranes (panel A) or 3T3 membranes (panel B), in the presence (black bars) or absence (white bars) of 30 μM GTPγS. Results shown are from a single experiment performed in duplicate.


A) HL 60 CELLS
[^{3}H] CHOLINE (DPM)
4000
3000
2000
1000
0
MEM
MEM + B 1
MEM + B 2
MEM + CYT
B) 3T3 CELLS
[^{3}H] CHOLINE (DPM)
1500
1000
500
0
MEM
MEM + B 1
MEM + B 2
MEM + CYT

PURIFICATION OF GST-RHO FROM *ESCHERISCHIA COLI* AND CHARACTERIZATION OF RHO REGULATED PLD ACTIVITY

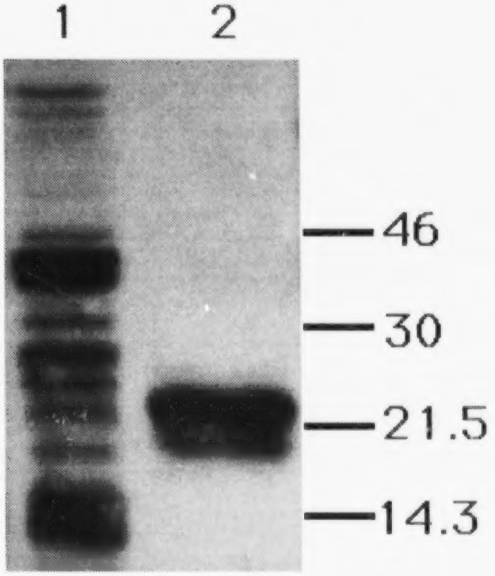
The involvement of GTP γS in leading to an increase in PLD activity implicates the involvement of G proteins. In the previous section, ARF was shown to substitute for the cytosolic requirement for the activation of membrane PLD in HL 60 and 3T3 cells. ARF seems not to be the only small G protein of the Ras superfamily that will activate PLD. Bowman *et al.* (1993) reported that in human neutrophils PLD activity is stimulated by Smg GDS, which is a GDP/GTP dissociation stimulator, and inhibited by Rho GDI which is a GDP/GTP dissociation inhibitor. These two findings demonstrated that one of the members of the Rho subfamily of small G proteins was also involved in stimulating PLD. Although these two GDP/GTP exchange proteins are capable of regulating several members of the small G protein families, further evidence of the Rho involvement in activating PLD was provided by Malcolm *et al.* (1994), who showed that membrane bound Rho A stimulated PLD in rat liver cells. Members of the Rho subfamily are involved in activation of NADPH oxidase, membrane ruffling, and actin polymerization (Ridley *et al.*, 1992).

We also studied the effect of Rho in modulating the activity of PLD in 3T3 and HL 60 cells. Recombinant Rho A was coupled to GST in a pGEX-2T vector, which was kindly provided by Dr A. Hall. Using a culture of *E. coli* , we isolated Rho A-GST by its ability to bind to glutathione Sepharose beads. To test for the purity of this preparation we performed Western blot analysis on the purified product using a monoclonal antibody against Rho A. The purified product contains only one major band of an apparent molecular weight of 21 kDa which is identified as Rho A (Figure 12).

To examine the effect of Rho A on PLD activity we incubated the recombinant protein with membranes from 3T3 and HL 60 cells in the

FIG. 12 WESTERN BLOT ANALYSIS OF RECOMBINANT RHO A PRODUCED AS A GST-FUSION PROTEIN IN *E. COLI.*

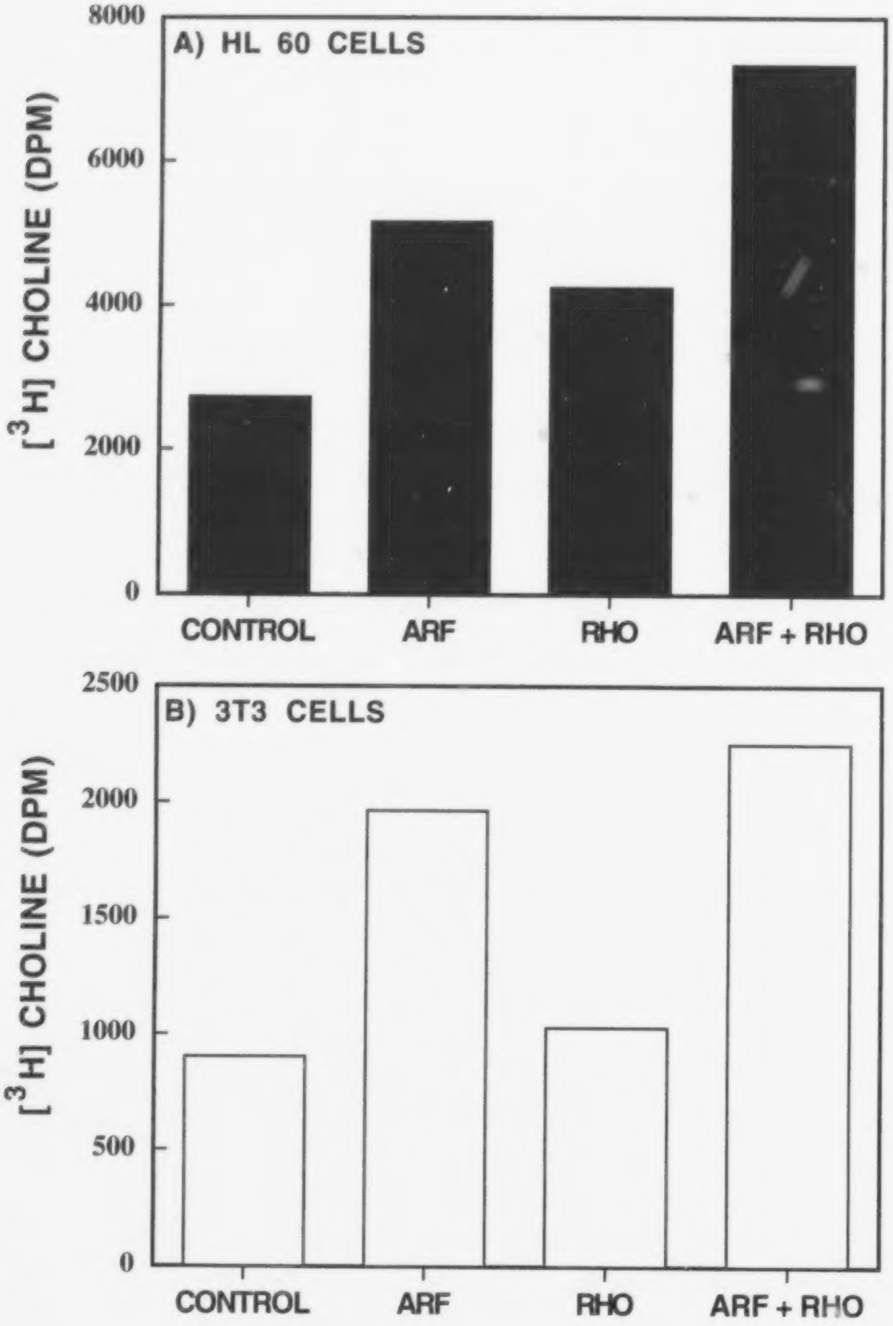
RhoA was expressed as a GST-fusion protein in *E. coli* as described in Materials and Methods. Samples taken from two steps during the purification process were subjected to SDS-PAGE on a 12 % gel and were then transferred onto nitrocellulose membrane for western blot analysis according to the procedure outlined in Materials and Methods, using a monoclonal antibody against Rho A. The cross-reactivity of this antibody with the protein(s) was visualized by ECL using a secondary antibody coupled to horse radish peroxidase . *Lane* 1, a sample from *E. coli* cell lysate prior to purification: *Lane* 2, sample from the purified product after thrombin cleavage of GST-RhoA bound to glutathione-coated sepharose beads.


1
2
46
30
21.5
14.3

presence of 30 μM GTP γS. Addition of Rho A (1 nM) to HL 60 membranes resulted in a 1.6 fold activation; in the same experiment addition of 25 nM ARF resulted in a 2-fold stimulation. Addition of both ARF and Rho A was found to activate PLD in an additive manner resulting in a 2.5 fold activation (Figure 13; panel A). Different results were obtained when 3T3 membranes were used as the source of PLD; in this case only ARF showed an activation but Rho A did not. In addition, ARF and Rho A did not show an additive effect, suggesting that the activation of PLD by small G proteins could be a cell specific-event. The higher PLD activity observed in the presence of both ARF and Rho A does not allow for a definite speculation about which of the two G proteins is the true activator of PLD. ARF has been implicated in membrane trafficking and endosome fusion. In HL 60 membranes, one possible mechanism by which the additive effect of ARF and Rho A occurs, could involve ARF as the cofactor required for fusion of the substrate micelle with the membranes containing PLD, leaving Rho A to be the candidate activator of PLD.

FIG. 13 EFFECT OF THE SMALL MOLECULAR WEIGHT G PROTEIN r Rho A ON PLD ACTIVITY IN HL 60 AND 3T3 CELLS.

Membranes from either HL 60 cells (panel A) or 3T3 cells (panel B) were prepared as described in Materials and methods. Twenty µg of membrane protein were incubated with 30 µM GTP γS alone (control), plus 25 nM ARF (purified product containing a mixture of myristoylated and non-myristoylated forms) or 1nM RhoA, alone or in combination. Results shown are representative of one of three experiments performed in duplicate.


A) HL 60 CELLS
[^{3}H] CHOLINE (DPM)
8000
6000
4000
2000
0
CONTROL
ARF
RHO
ARF + RHO
B) 3T3 CELLS
[^{3}H] CHOLINE (DPM)
2500
2000
1500
1000
500
0
CONTROL
ARF
RHO
ARF + RHO

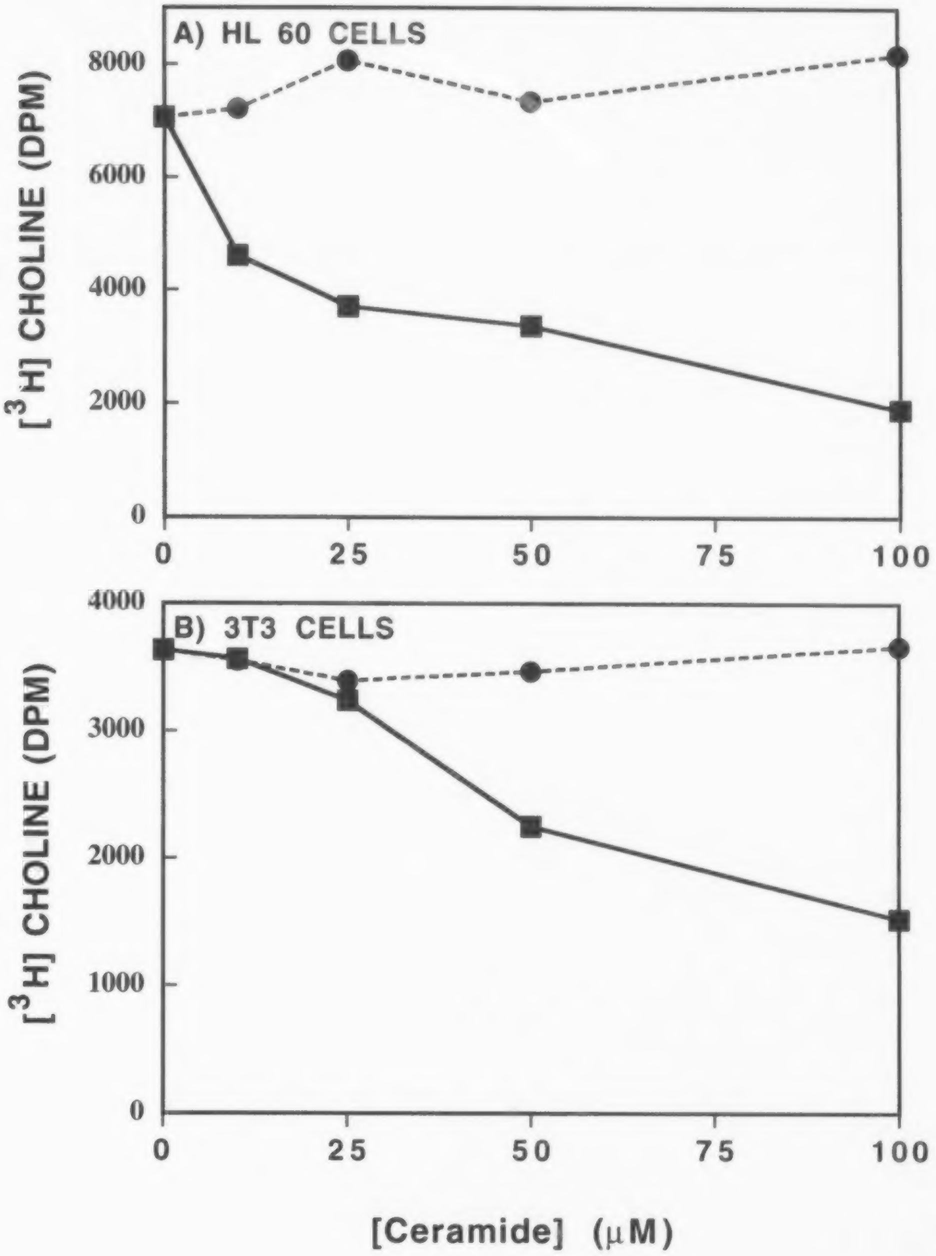
THE EFFECTS OF CELL PERMEABLE C 2 AND C 6 CERAMIDES ON THE RECONSTITUTED AND SMALL MOLECULAR WEIGHT G PROTEIN REGULATED PLD ACTIVITY

Over the past decade increasing experimental evidence indicates that sphingolipids are the source of important lipid second messengers which can modulate several biological events. Breakdown of sphingolipids by sphingomyelinase gives rise to ceramide. Many reports have shown that this breakdown can be signal mediated through the action of TNFα on several cell types including HL 60 cells (Kim *et al.*, 1990). To study the role of ceramides in modulating signal transducing pathways, investigators use short chain ceramide analogs which are more soluble and thus easier to handle. They are also readily permeable through the cell surface when compared to their natural counterparts which are usually between 16 and 18 carbons long. In our laboratory using C-2 and C-6 ceramides, Gomez-Munoz *et al.* (1994) reported that ceramides inhibited both agonist-stimulated and GTPγS dependent PLD activity in intact and permeabilized rat fibroblasts, respectively. This inhibition was time and concentration dependent with a maximal inhibition of [^{3}H]phosphatidylethanol production at 50 μM of either C-2 or C-6 ceramide.

To follow on this evidence, the effects of ceramides on both the reconstituted and cell free PLD assay were studied. Both membranes and cytosol from either HL 60 or 3T3 cells were used in the presence of 30 μM GTP γ S to reconstitute the PLD activity. C-2 but not C-6 ceramide inhibited the GTP γ S dependent PLD activity in both cell lines (Figure 14; panels, A and B). The maximum inhibition was observed at 100 μM (reaching a plateau at 200 μM; result not shown), whereas C-6 at the same concentration had no effect on PLD activity. In both 3T3 and HL 60 cells there was a considerable amount of activity that was not inhibited by C-2 ceramide which may be due to multiple isoforms of PLD with different sensitivities to ceramide. The lack of inhibition observed with the use of C-6 was not due to loss of bioactive ability due to degradation because C-6 from different sources

FIG. 14 EFFECT OF C2 AND C6 CERAMIDES ON THE RECONSTITUTED *IN VITRO* PLD ACTIVITY.

Membranes plus cytosol from either HL 60 cells (panel A) or 3T3 cells (panel B) were assayed for PLD activity according to the section described in Materials and Methods. The graphs depict the release of [^{3}H] choline in dpm versus increasing concentrations of cell-permeable C-$_2$ (black squares) and C-$_6$ (black circles) ceramides. Results shown are representative from one of two experiments performed in duplicate.

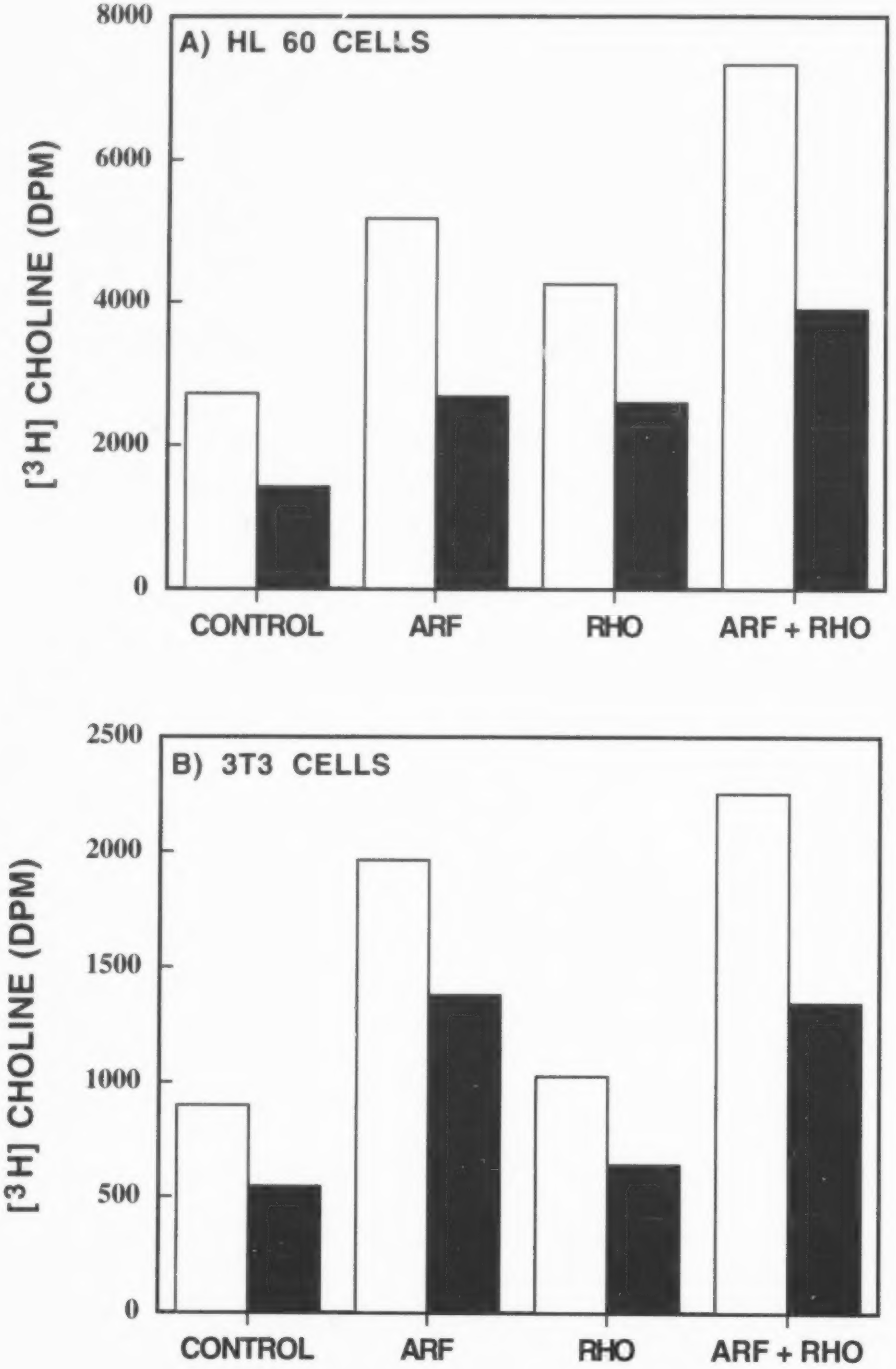

A) HL 60 CELLS
B) 3T3 CELLS
[^{3}H] CHOLINE (DPM)
0
2000
4000
6000
8000
1000
3000
0
25
50
75
100
[Ceramide] (μM)

had exactly the same response. The lack of inhibition could be attributed to solubility problems as C-$_6$ reportedly is less soluble than C-$_2$; this was examined further in the following section. The effects of C-$_2$ ceramide were further studied in the cell free assay system where membrane bound PLD could be activated in the presence of the recombinant small molecular weight G proteins ARF and Rho A (Figure 15). Addition of 100 μM C-$_2$ ceramide resulted in a 52% inhibition of ARF stimulated PLD activity, 62% inhibition of RhoA stimulated PLD activity, and 52% inhibition of ARF plus RhoA stimulated PLD activity. Comparable results were obtained also when membranes from 3T3 cells were assayed.

This is the first report that provides evidence that C-$_2$ ceramide inhibits the reconstituted, GTPγS dependent, membrane bound PLD activity from HL 60 and 3T3 cells. Furthermore, C-$_2$ ceramide blocks half the stimulation observed in the presence of ARF, Rho A, and the additive effect of both ARF and RhoA which has also been observed by Siddiqi *et al.* (1995). The amount of PLD activity that is not inhibited by ceramide could be due to a separate isoform of the enzyme that remains unresponsive to this inhibitory effect and could probably be used as a useful tool to help characterize a new member of the PLD isoform family. Further evidence that multiple isoforms of PLD enzyme or multiple pathways that stimulate the same or different isoform(s) could exist within a cell is provided by the 50 % inhibition of PLD activity in the presence of 3 mM GDP βS as shown in Figures 6 and 7 for both cell lines. Taken together these results indicate that in 3T3 and HL 60 cells there is a PLD activity which is independent of ceramides and /or small molecular weight G proteins.

FIG. 15 **EFFECT OF C2 CELL-PERMEABLE CERAMIDE ON THE THE SMALL MOLECULAR WEIGHT G PROTEIN-STIMULATED PLD ACTIVITY.**

Membranes from either HL 60 cells (panel A) or 3T3 cells (panel B) were prepared as described in Materials and Methods. Twenty μg of membrane protein were incubated with 30 μM GTP γS (control) or, 30 μM GTP γS plus 25μM ARF (ARF) or, 30 μM GTP γS plus 1 nM RhoA (RHO) or, 30 μM GTP γS plus 25μM ARF and 1nM RhoA (ARF + RHO) (white bars). The effect of 100 μM C-$_2$ ceramide on these activities is depicted in the black bars. Results shown are from a representative experiment from three independent experiments performed in duplicate.


A) HL 60 CELLS
[3H] CHOLINE (DPM)
8000
6000
4000
2000
0
CONTROL
ARF
RHO
ARF + RHO
B) 3T3 CELLS
[3H] CHOLINE (DPM)
2500
2000
1500
1000
500
0
CONTROL
ARF
RHO
ARF + RHO

DIFFERENTIAL PRESENTATION OF C-2 AND C-6 CERAMIDES TO THE CELL FREE PLD ASSAY

The effect of C-2 and C-6 ceramides on the membrane PLD activity from HL 60 and 3T3 cells was examined in the previous section. Since we were only successful to reproduce the inhibitory effect of C-2, but not of C-6, on the activation of PLD in either cell line, and in an attempt to circumvent the solubility problem encountered with the C-6 compound, a different strategy of presenting the ceramides to the assay was employed. The following series of experiments were also designed to probe for some answers to the mechanism (s) by which ceramides confer their inhibitory effects on PLD.

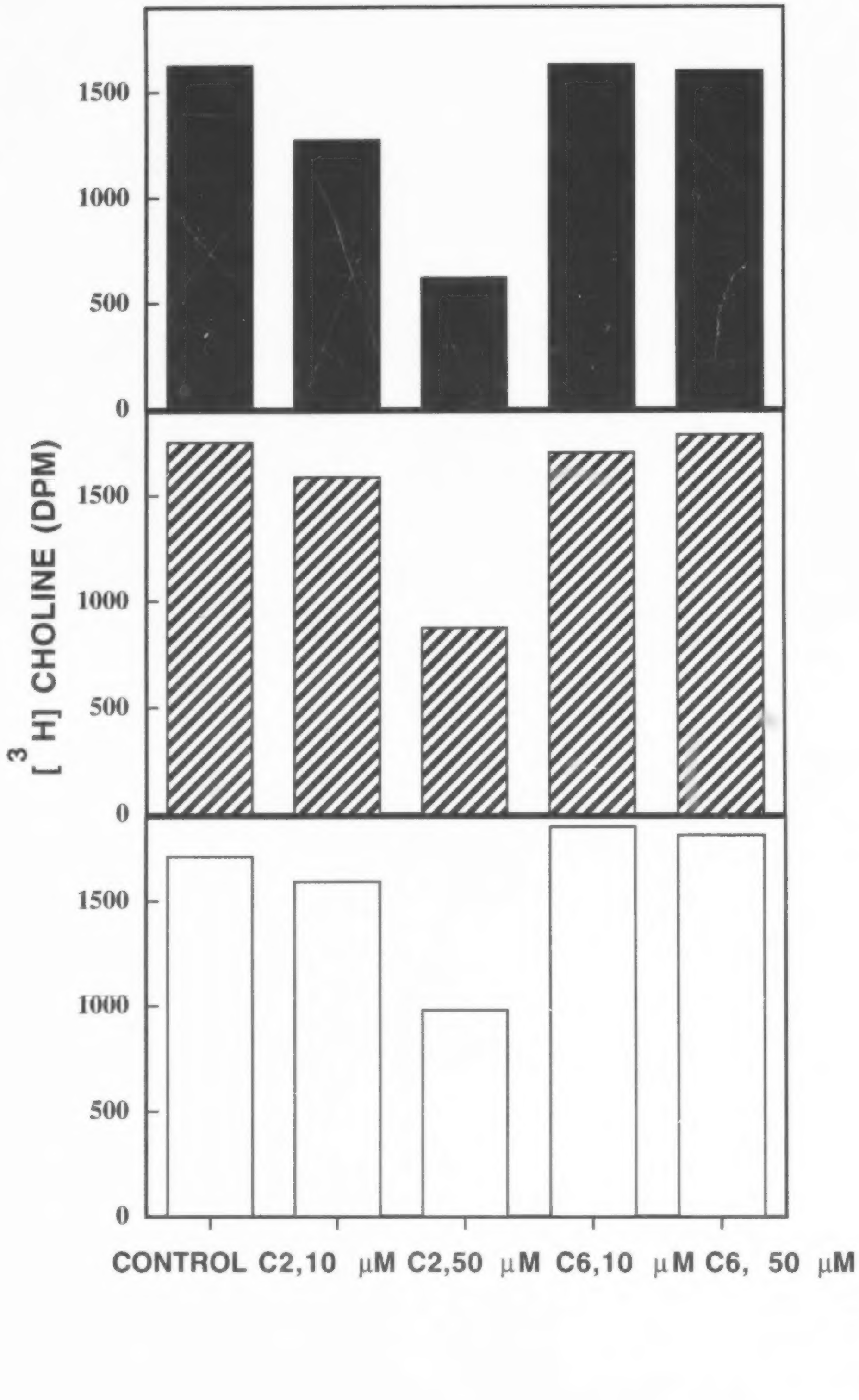
Both C-2 and C-6 ceramides were dissolved in DMSO at a stock concentration of 10 mM. In the previous section, ceramides were added to the assay mixture after a 1 in 1000 dilution with distilled water. During this dilution, C-6 was not completely soluble and the 10 µl addition to the assay probably contained less than the expected concentration. One of the approaches to this problem was to facilitate the solubility of ceramides in the assay by adding a phospholipid liposome as a ceramide carrier. This would also enable us to target the ceramide directly to the membranes where PLD activity is detected, since the ceramide-liposome should normally fuse with the lipid containing membranes. As the ceramide carrier we used PE, which is a component of the substrate liposome in the assay. One-half of the overall PE concentration (68.8 µM) in the assay was used along with either C-2 or C-6 ceramide to construct a PE-ceramide liposome by probe sonication. The 3T3 membranes were preincubated with this liposome for 10 minutes and the reaction was started with the addition of the substrate liposome that contained the remaining half of PE.

To explore the possibility that the inhibitory effect of ceramide could be due to some counter action to the PIP_2 requirement in the PLD assay, ceramide was included in the substrate liposome. This was achieved by drying all the lipid components of the liposome along with the ceramide, creating liposomes by probe sonication and starting the assay by adding this substrate to the 3T3 membranes containing PLD.

The resulting activities of the ceramide-PE liposome (depicted in Figure 16; top panel, black bars) showed no significant difference from the results observed when ceramides where added with no phospholipid carrier (Figure 16; bottom panel, white bars). Furthermore, the presence of ceramide in the substrate liposome (Figure 16; middle panel, hatched bars) also made no difference to the extend of inhibition of PLD activity. In all cases C-$_6$ ceramide was completely inactive.

FIG. 16 EFFECTS OF DIFFERENTIAL PRESENTATION OF C-2 AND C-6 CERAMIDE TO PLD FROM 3T3 MEMBRANES.

Membranes from 3T3 cells were prepared as outlined in the Materials and Methods section. The top panel (black bars) represents results from assays in wich a liposome containing 68.8 µM PE and either 10 or 50 µM of C-2 or C-6 ceramide were preincubated for 10 minutes with 3T3 membranes. The middle panel (hatched bars) represents results from experiments where the specified concentration of C-2 or C-6 ceramide was included in the substrate liposome. The bottom panel depicts results from experiments where ceramides where added to the assay after the appropriate dilution from a stock concentration dissolved in DMSO. Control refers to the addition of DMSO carrier at a final concentration in the assay of 0.1%. Results are representative from one of two experiments performed in duplicate.


[^{3}H] CHOLINE (DPM)
0
500
1000
1500
CONTROL
C2,10 µM
C2,50 µM
C6,10 µM
C6, 50 µM

INVESTIGATION OF THE INVOLVEMENT OF PKC IN REGULATING PLD USING THE CELL FREE ASSAY

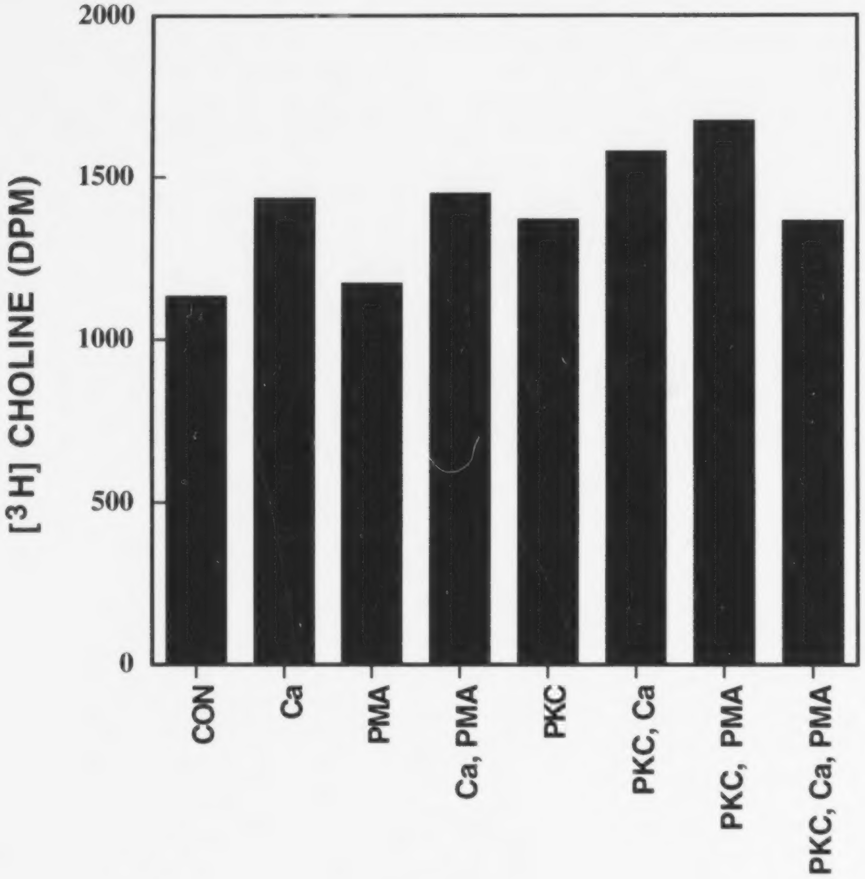
Many reports have implicated the involvement of PKC in regulating PLD activity in whole cells (see Introduction). Conricode *et al.* (1992) have also shown that PKC regulates PLD in a cell free system. In their report, [^{14}C]choline-labelled membranes from Chinese hamster lung fibroblasts were incubated with purified PKC from rat brain. In the presence of the DAG analog PMA, a 2-3 fold activation of PLD was observed, which was sustained even at the presence of high concentrations of apyrase. These results suggested that not only that the PMA stimulation of PLD requires PKC, but also that this activation is achieved through a phosphorylation -independent mechanism.

In light of this evidence, we attempted to reproduce this effect of PKC on the regulation of PLD in membranes from 3T3 cells. Once this stimulation could be established, ceramides would be used to find out more about PKC and small molecular weight G proteins as either parallel or converging pathways involved in the activation of PLD. The effect of PKC from rat brain was investigated using the cell free PLD assay. Protein kinase C failed to activate PLD in our system (Figure 17). No significant difference from the control was detected when PKC was added to membranes in the presence of ATP (1 mM), Ca^{2+} (4 mM), and PMA (100 nM), which are all known activators of the kinase. The lack of activation could be due to loss of PKC activity since we never performed any positive control experiments of our own but rather relied on the information about the activity of the PKC preparation given to us from Dr. Lord's laboratory personel.

The lack of activation of PLD in the presence of purified PKC in our assay system does not exclude a PKC involvement in the PLD pathway because in a recent report by Yeo and Exton (1995) Ro-31-8220 , a potent inhibitor of PKC, inhibited in a dose-dependent manner the stimulation of PLD by epidermal growth factor in Swiss 3T3 cells.

FIG. 17 THE EFFECT OF RAT BRAIN PKC ON THE ACTIVATION OF MEMBRANE BOUND PLD FROM 3T3 CELLS.

Membranes from 3T3 cells were assayed for PLD activity according to the procedure outlined in Materials and Methods. Purified PKC (a gift from Dr. Lord, Department of Immunology, University of Birmingham, UK) was incubated with membranes along with a combination of Ca^{2+} (40 mM), and PMA (100 nM). All assays were performed in the presence of ATP (1mM). Results shown are representative of one of two independent experiments performed in duplicate.


[^{3}H] CHOLINE (DPM)
2000
1500
1000
500
0
CON
Ca
PMA
Ca, PMA
PKC
PKC, Ca
PKC, PMA
PKC, Ca, PMA

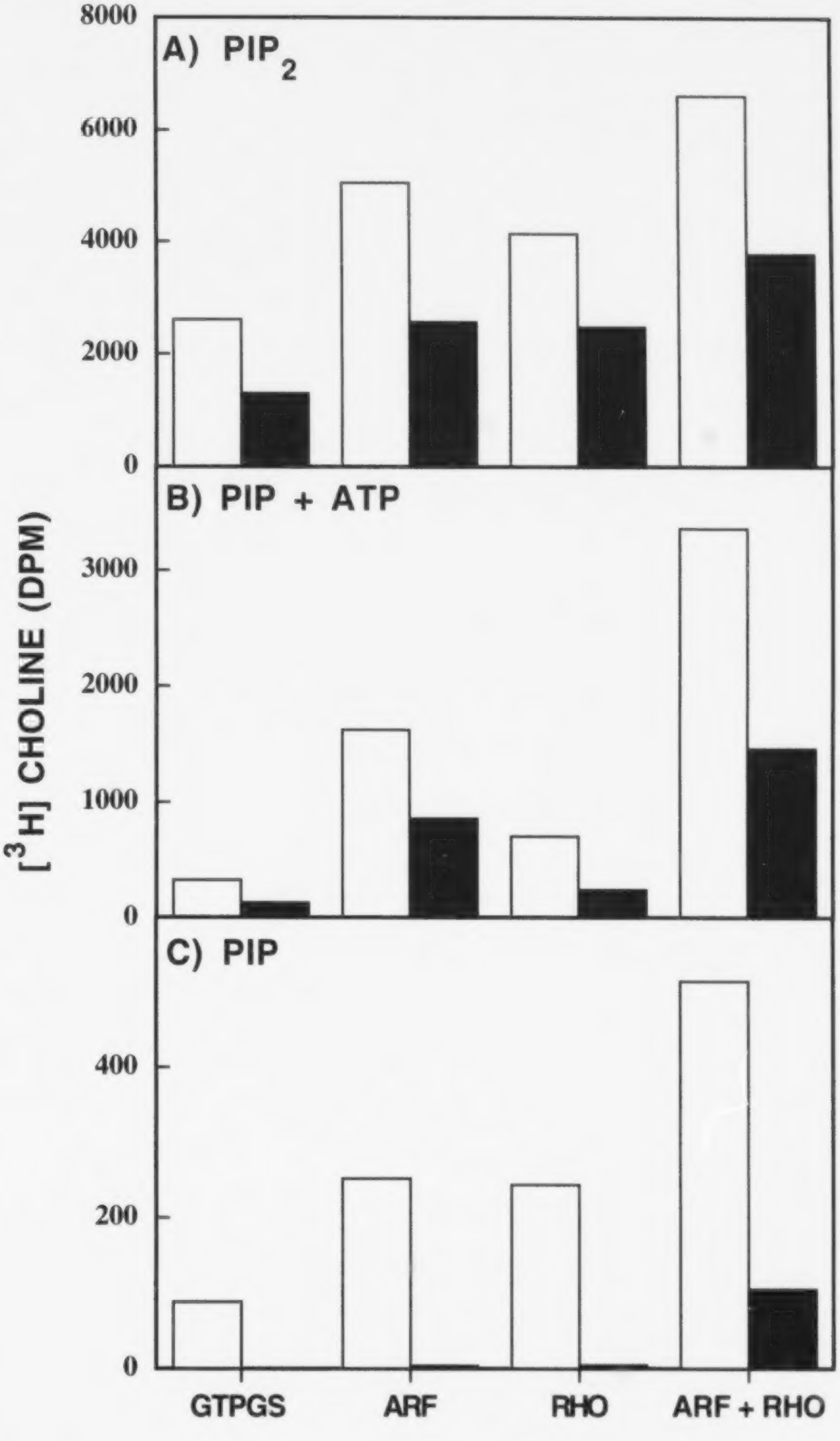
PHOSPHATIDYLINOSITOL 4-PHOSPHATE CAN SUBSTITUTE FOR THE PIP_2 REQUIREMENT FOR DETECTION OF PLD ACTIVITY IN THE PRESENCE OF SMALL MOLECULAR WEIGHT G PROTEINS AND ATP

The detection of PLD activity, when assayed with exogenous substrate liposomes, requires the presence of PIP_2 (Brown *et al.* 1993). This absolute requirement for PIP_2 was also confirmed in our studies with both 3T3 and HL 60 cells (see earlier section). Chong *et al.* (1994) showed that Rho regulates the activity of a PIP-5 kinase in mouse fibroblasts and plays a critical role in regulating cellular PI 4-5 P_2 levels. Since cellular PIP_2 levels have been correlated with cell adhesion, i.e. adherent cells have high PIP_2 levels whereas suspended cells have low PIP_2 levels, the authors predict that PIP_2 may mediate the effects of Rho on the cytoskeleton. Consequent to that information, we attempted to stimulate production of PIP_2 using intracellular enzyme machinery in order to find out if it was possible to substitute for the requirement of exogenous PIP_2 and thus observe an increase in PLD activity.

Membranes from HL 60 cells were incubated with small molecular weight G proteins (ARF, Rho) and substrate liposomes containing phosphatidylinositol-4 phosphate (50 μM) (an arbitrary concentration 4 times higher than that of the PIP_2 in the control condition) in the presence (Figure 18; panel B) or absence of ATP (1 mM) (Figure 18; panel C). This concentration was chosen so that the presence of excess substrate concentration under the appropriate conditions would drive the synthesis of PIP_2. The activity of PLD under those conditions was compared to control experiments where PIP_2 (12 μM) was present in the exogenous substrate liposome (Figure 18; panel A). Addition of either ARF or Rho in the presence of PIP and ATP (Figure 19; panel B, white bars) was not sufficient for recovery of the PLD activity observed when PIP_2 was included (Figure 18; panel A; white bars). About 50 % of the activity was recovered when ARF and Rho were combined indicating that they may act in a synergistic fashion to facilitate the

FIG. 18 ARF AND RHO CAN PARTIALLY SUBSTITUTE THE EXOGENOUS PIP$_2$ REQUIREMENT OF PLD ACTIVITY IN THE PRESENCE OF PIP AND ATP.

Membranes from HL 60 cells were collected and assayed for PLD activity according to the procedures outlined in the Materials and Methods section. Panel A represents results obtained when PIP$_2$ (12 µM) was included in the substrate liposome. In panel B, the PIP$_2$ present in the substrate liposome was substituted by phosphatidylinositol 4-phosphate (50 µM) in the presence of ATP (1mM). Panel C depicts results from assays where phosphatidylinositol 4-phosphate (50 µM) was present in the substrate liposome in the absence of ATP. In all panels white bars represent assays containing 30 µM GTPγS, black bars represent assays where the effect of C-$_2$ ceramide (100 µM) in addition to 30 µM GTPγS is tested. Results are representative from one of two experiments performed in duplicate.


A) PIP$_2$
B) PIP + ATP
C) PIP
[^{3}H] CHOLINE (DPM)
8000
6000
4000
2000
0
3000
2000
1000
0
400
200
0
GTPGS
ARF
RHO
ARF + RHO

activation of PLD. The activity observed in the presence of ARF and Rho was ATP dependent because in its absence the resulting PLD activity was minimum (Figure 18; panel C, white bars). In the same experiment the effect of C-2 ceramide (100 μM) was also examined (Figure 18; all panels, black bars). C-2 ceramide inhibited the activation of PLD which was due to the combined action of ARF and Rho on the synthesis of PIP_2.

In summary, the absolute requirement for PIP_2 in observing PLD activity, using an exogenous substrate, can be partially bypassed in the presence of both ARF and Rho whose concerted action can stimulate cellular machinery to synthesize PIP_2 from exogenous PIP and ATP, presumambly through the action of phosphatidylinositol 4-phosphate 5-kinase. It was suspected that the presence of Rho alone should have been sufficient in stimulating the synthesis of PIP_2 and thus activate PLD but this did not seem to be the case. The finding that both ARF and Rho were required to partially recover the PLD activity may suggest that ARF could be required for fusion of the PIP-containing liposome with membranes where the PIP 4-phosphate kinase is found whereas Rho could be involved in the stimulation of the kinase activity itself. Finally, the incomplete recovery of PLD activity in the presence of PIP and ATP could be due to levels of newly synthesized PIP_2 not reaching a concentration of 12 μM which was shown to be maximal for PLD stimulation (actual PIP_2 production under this conditions was not measured).

The results presented here show that the activation of PLD by small molecular weight G proteins seems to be a direct effect, yet the finding that ARF and Rho could also participate in the production of PIP_2, a potent cofactor for PLD stimulation as well as α-actinin polymerization, may link PLD activation to changes in the cytoskeleton and therefore changes in cell shape.

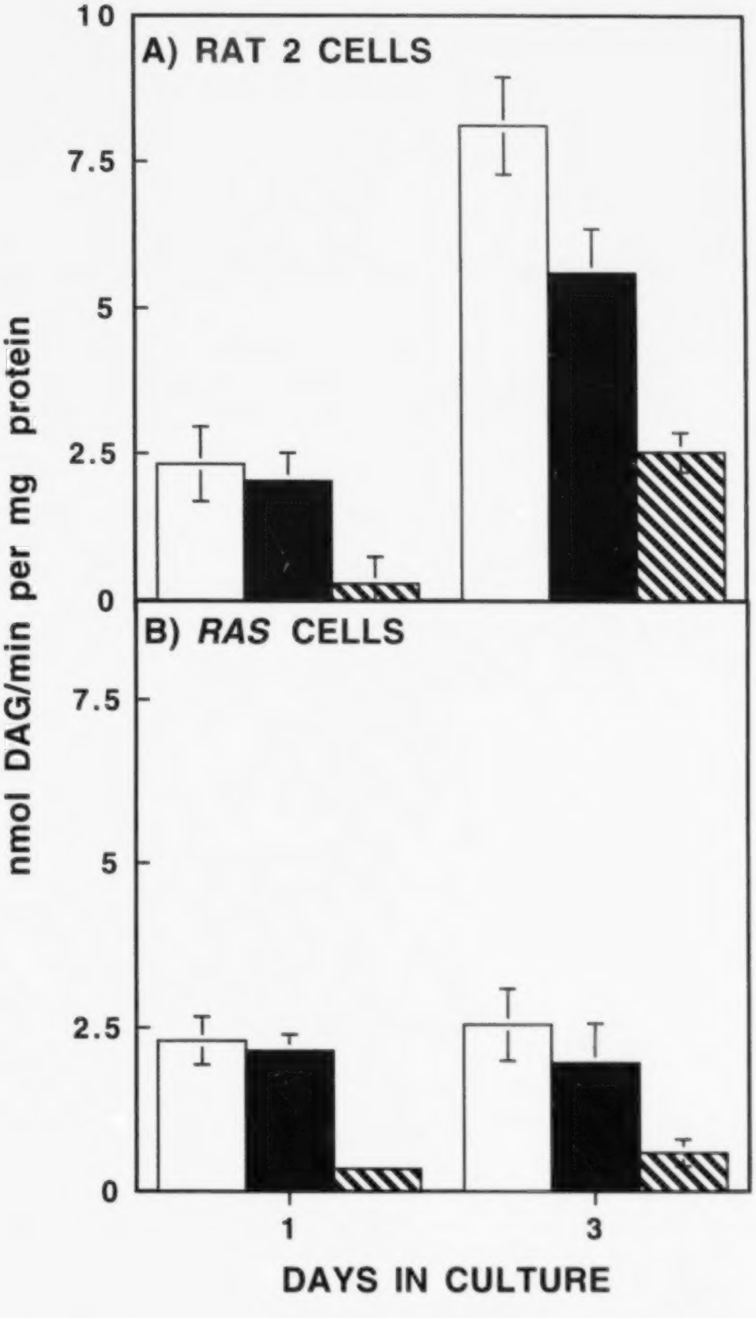
STUDIES ON THE Mg^{2+}-DEPENDENT PHOSPHATIDATE PHOSPHOHYDROLASE (PAP-1) ACTIVITY IN CONTROL AND *RAS* -TRANSFORMED FIBROBLASTS

Phosphatidate (PA) and DAG have been shown to play a central role as second messengers in many signal transduction pathways (see Introduction). Phosphatidate can be metabolized readily to DAG through the action of PAP. Recently our group reported that the activity of PAP-2, an integral plasma membrane enzyme which is NEM-insensitive, does not require Mg^{2+} , and is thought to be involved in signal transduction processes. This activity decreased with time in culture in *ras* -transformed and *fps* -transformed fibroblasts compared to control fibroblasts (Martin *et al.*, 1993). In addition, stimulation of either *ras* - or *fps* -fibroblasts with PMA or serum resulted in lower DAG generation and decreased ratios of DAG relative to PA when compared to non-transformed cells. These findings imply that PAP-2 in transformed cells is unable to counteract increases in PA, by metabolizing it, due to agonist stimulation. In a subsequent study (Martin *et al.*, 1995), the relative cellular masses of DAG and PA were measured in *ras* -, *fps* -, and control fibroblasts. Control fibroblasts stop growing by day 3 in culture as they become contact inhibited whereas transformed cells continue to grow in an uncontrolled fashion. Phosphatidate levels coincided with the growth trend of the transformed cells. The PA mass of the *ras* -transformed fibroblasts was higher on day 1 and increased 5 to 6 fold on day 3 compared to control PA levels which not only were lower but also decreased by 28% between day 1 and day 3.

In light of these observations we set out to determine the activity of PAP-1, which is thought to be mainly involved in glycerolipid synthesis, in control and oncogenically transformed fibroblasts. The activity profile of PAP-1 in *ras* -transformed and control fibroblasts with time in culture is illustrated in figure 19. No significant difference in PAP-1 activity was observed on day 1 between the two lines. However the membrane bound

FIG. 19 EFFECT OF TIME IN CULTURE ON THE ACTIVITY OF THE Mg^{2+}-DEPENDENT PAP-1 IN CONTROL AND *RAS* -TRANSFORMED FIBROBLASTS.

Fibroblasts were cultured for the time indicated and permeabilized with digitonin according to the Materials and Methods. The leaking of cytosolic proteins was monitored by measuring the LDH activity in the separated cytosol and the cell ghosts which were considered to represent the membrane fraction. PAP-1 activity was measured according to the procedure outlined in the Materials and Methods. The specific activity of the NEM-sensitive PAP-1 is expressed relative to the total protein content of the fibroblasts. The results are means ± sd from three independent experiments. (white bars, total PAP-1 activity; black bars, cytosol associated activity; hatched bars, membrane associated activity)


A) RAT 2 CELLS
B) *RAS* CELLS
nmol DAG/min per mg protein
10
7.5
5
2.5
0
7.5
5
2.5
0
1
3
DAYS IN CULTURE

form of PAP-1, which is thought to be the physiologically active form of PAP-1 activity, and the total i.e. membrane plus cytosol PAP-1 activity in control cells increased by 3-fold on day 3. In contrast, the PAP-1 activity of *ras* -transformed cells remained unchanged from that observed on day 1. The activity of PAP-1 in *fps* cells was similar to the other two cell lines on day 1 and increased 1.4 fold on day 3 (Figure 20).

Phosphatidate phosphohydrolase-1 also has the additional feature from PAP-2 of being able to translocate in rat hepatocytes from the cytosol, where it is normally found, to membranes in response to high levels of PA, fatty-acyl CoA esters, and long-chain fatty acids such as oleate (Gomez-Munoz *et al.*, 1992) . We also tested the ability of PAP-1 to translocate to membranes in rat fibroblasts in response to oleate. Figure 21 depicts the effects of increasing concentrations of oleate on the subcellular distribution of PAP-1 in control and *ras* -transformed fibroblasts. Translocation of PAP-1 in response to oleate was only observed in rat 2 cells and was completely absent in *ras* -fibroblasts where PAP-1 remained cytosolic even at concentrations of oleate of up to 1 mM. Increasing concentrations of oleate were inhibitory to the total activity of PAP-1. Unlike rat hepatocytes, where the presence of oleate resulted in a 4-fold rise of PAP-1 activity, the PAP-1 activity of fibroblasts was inhibited in the presence of oleate. This inhibition of PAP-1 in non hepatic tissues has also been observed by others (Cascales *et al.*, 1984). Since the translocation of PAP-1 to the membranes in hepatocytes, can be facilitated by the action of insulin (Pitner *et al.*, 1985) and counteracted by glucagon and cAMP (Pitner *et al.*, 1985 and Butterwith *et al.*, 1984, respectively), it may be considered to be due to charge attraction. We attempted to induce translocation of PAP-1 by increasing the levels of PA in the two cell lines using PMA and FCS which stimulate PLD activity by two independent mechanisms. PAP-1, in both cell lines, remained cytosolic in the presence of these agonists, suggesting that either PAP-1 is not sensitive to changes of PA levels in the membrane microenvironment or that PA levels rise in membrane compartments not accessible to PAP-1. Translocation in response to 0.5 mM oleate (18:1) was only observed

FIG. 20 EFFECT OF TIME IN CULTURE ON THE ACTIVITY OF THE Mg^{2+}-DEPENDENT PAP-1 IN *FPS* FIBROBLASTS.

Fibroblasts were cultured for the time indicated and permeabilized with digitonin (0.25 mg/ml) in HEPES (10mM) and DTT (1mM) according to the section in Materials and Methods. The leaking of cytosolic proteins was monitored by measuring the LDH activity in the separated cytosol and the cell ghosts which were considered to represent the membrane fraction. PAP-1 activity was measured according to the procedure outlined in the Materials and Methods. The specific activity of the NEM-sensitive PAP-1 is expressed relative to the total protein content of the fibroblasts. The results are representative from one of two experiments performed in triplicate. (white bars, total PAP-1 activity; black bars, cytosol associated activity; hatched bars, membrane associated activity)

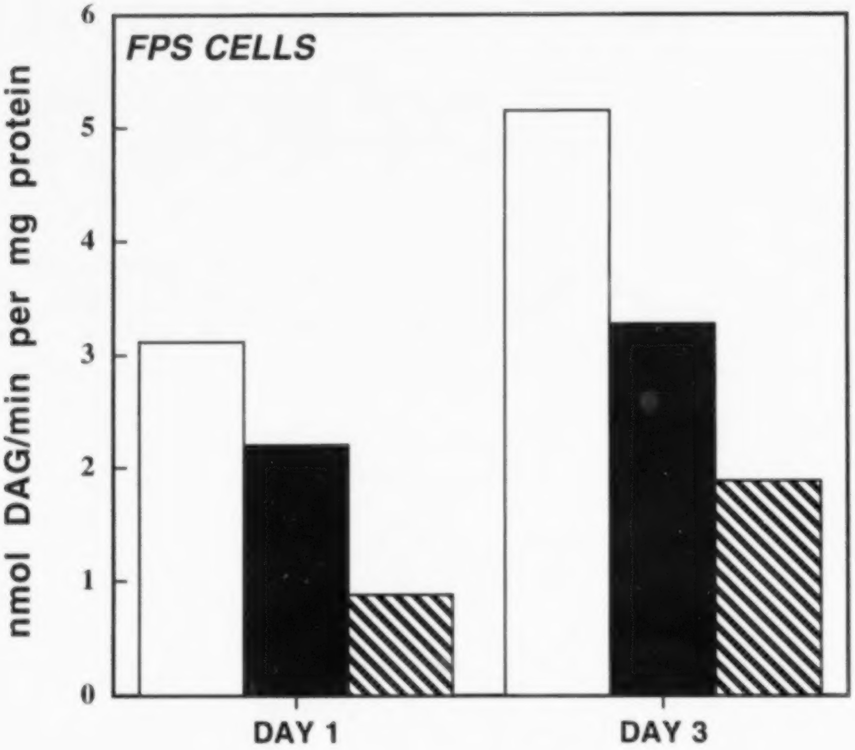
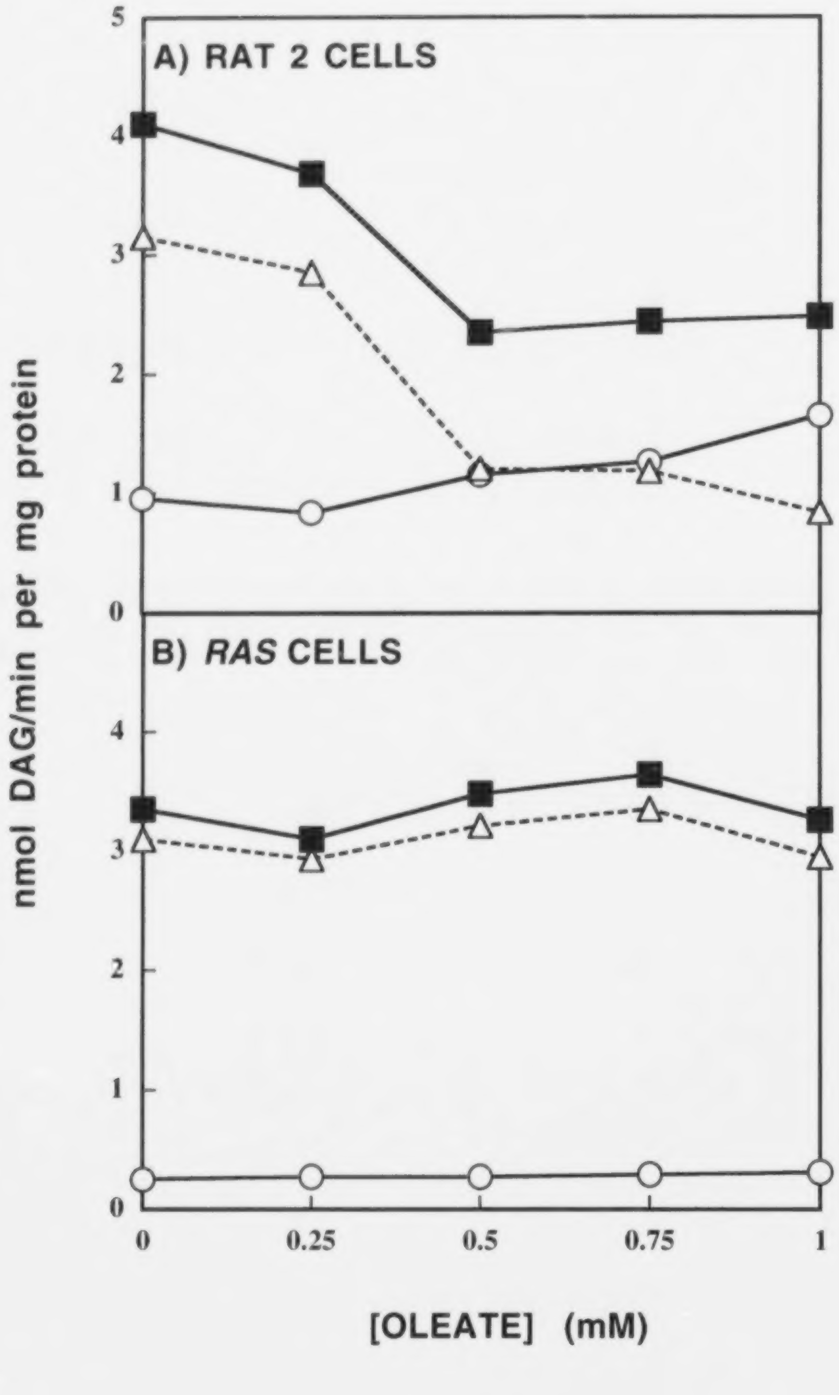

FPS CELLS
nmol DAG/min per mg protein
0
1
2
3
4
5
6
DAY 1
DAY 3

FIG. 21 **EFFECT OF OLEATE ON THE TRANSLOCATION OF Mg^{2+}-DEPENDENT PAP-1 IN CONTROL AND *RAS* -TRANSFORMED FIBROBLASTS.**

Fibroblasts were cultured according to the section outlined in Materials and Methods. The cells were made quiescent for 2 hours with DMEM containing 2% BSA. Oleate (18:1) at the indicated concentrations was added to the medium of quiescent cells for 15 minutes and the cells were then permeabilized with digitonin (0.25 mg/ml) in HEPES (10mM) and DTT (1mM) to separate cytosol from membranes. The specific activity of the NEM-sensitive PAP-1 is expressed relative to the total protein content of the fibroblasts. The results are representative of one of three independent experiments performed in duplicate. (black squares, total PAP-1 activity; white triangles, cytosol associated activity; white circles, membrane associated activity).

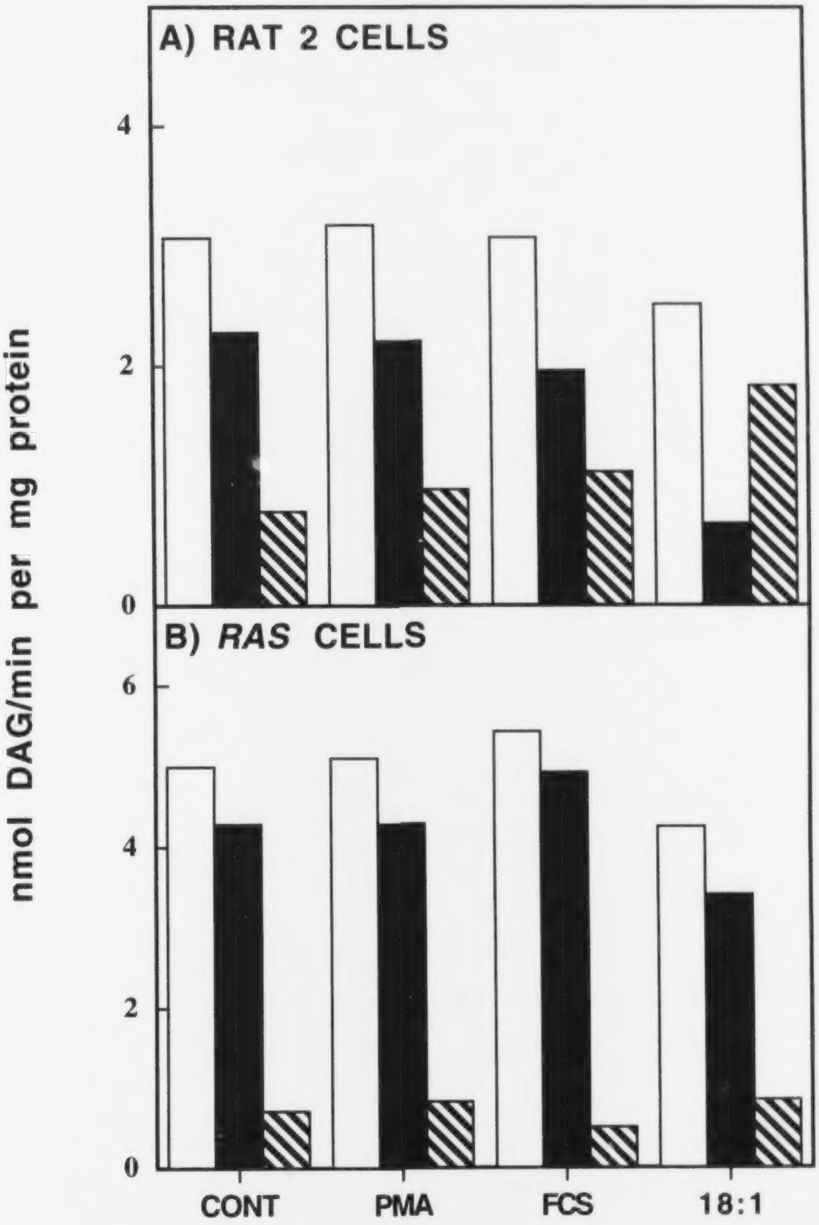

A) RAT 2 CELLS
B) *RAS* CELLS
nmol DAG/min per mg protein
[OLEATE] (mM)
0
1
2
3
4
5
0
0.25
0.5
0.75
1

in control fibroblasts, 73% of the total PAP-1 at this condition was found at the cell ghosts; whereas, in *ras* -transformed cells the majority of PAP-1 activity (80%) remained in the cytosol (Figure 22).

The PAP-1 activity profile in the two cell lines reported here completes the finding that both PAP activities are decreased in the transformed fibroblasts. Unlike control fibroblasts, PAP-1 in *ras* -transformed fibroblasts did not translocate to the membranes in response to oleate. PAP-1 could participate in signal transduction by translocating to the membranes in responce to increasing PA levels and aid in PA metabolism. Taken together the results presented in this section strengthen the possibility that the increased PA levels observed in *ras* -transformed but not in control fibroblasts can be due to the decreased activities of both PAP-1 and PAP-2.

FIG. 22 EFFECT OF FCS, PMA AND OLEATE ON THE TRANSLOCATION OF Mg^{2+}-DEPENDENT PAP-1 IN CONTROL AND *RAS* -TRANSFORMED FIBROBLASTS.

Fibroblasts were cultured according to the section outlined in Materials and Methods. The cells were made quiescent for 2 hours with DMEM containg 2% BSA. PMA (200 nM), or FCS (10%), or Oleate (18:1) (0.5 mM) was added to the medium of quiescent cells for 15 minutes and the cells were then permeabilized with digitonin (0.25 mg/ml) in HEPES (10mM) and DTT (1mM) to separate cytosol from membranes. The specific activity of the NEM-sensitive PAP-1 is expressed relative to the total protein content of the fibroblasts. The results are from one experiment performed in triplicate. (white bars, total PAP-1 activity; black bars, cytosol associated activity; hatched bars, membrane associated activity).


A) RAT 2 CELLS
B) RAS CELLS
nmol DAG/min per mg protein
0
2
4
0
2
4
6
CONT
PMA
FCS
18:1

DISCUSSION AND SUGGESTIONS FOR FURTHER EXPERIMENTS

The objectives of the work presented in this thesis were two fold. First, the cell free assay method for monitoring PLD activity (Brown *et al.*, 1993) was employed in HL 60 and 3T3 cells in order to characterize this enzyme activity in both cell systems. Second, the effects of two small molecular weight G-proteins, ARF and RhoA, in modulating PLD activity was investigated. In the case of 3T3 fibroblasts, this is the first report that shows an activation of PLD by small molecular weight G-proteins. In addition this is also the first report that provides evidence that the ARF and RhoA regulated PLD activity is inhibited by ceramide in HL 60 and 3T3 cells.

The initial experiments dealt with establishing the cell free PLD assay in our laboratory in both cell systems. PLD activity was detectable in membranes and cytosol obtained from HL 60 or 3T3 cells. PLD activity was stimulated when cytosol and membranes were combined in the presence of GTPγS (4-fold in HL 60 and .5 fold in 3T3 cells). No significant difference in activity was observed when GTPγS was included in assays containing either cytosol or membranes alone, indicating that a GTPγS sensitive factor existed in the cytosol which was able to stimulate membrane-bound PLD activity. The cytosolic factor required for activation of PLD exerted its effect in a concentration-dependent manner and was probably expressed in a variety of tissues since cytosol from HL 60 cells could activate membrane-bound PLD from 3T3 cells and vice versa. (This cytosolic factor has been identified as ARF by two groups independently, see Introduction). These results are in accordance with the ones observed from Brown *et al.*, (1993) and Cockcroft *et al.*, (1994) who used HL 60 cells as a model system for studying PLD activity.

Vanadate, a tyrosine-phosphate phosphatase inhibitor, abolished PLD activity when added to the PLD assay, although Bourgoin *et al.* (1992) had shown that in its presence PLD activity is increased in HL 60 cells. The results indicate that vanadate has a direct effect on the PLD rather than a change in the phosphorylation state of the enzyme since vanadate was neither present in the collection buffer nor during cell disruption by sonication where it would confer physiological changes in the cell. It is also possible that since PLD exists as a number of isoforms that vanadate could have isoform-specific effects. Addition of vanadate during preparation of membranes and cytosol rather than its addition to the assay could settle the discrepancy observed in PLD activities.

The membrane-bound PLD from fibroblasts was further characterized using the cell-free assay. The membrane bound enzyme activity (in 3T3 and rat 1 cells) was unaffected when the divalent metal ions Ca^{2+} and Mg^{2+} were absent, although Cockcroft *et al.*, (1994) reported that Ca^{2+} could modulate PLD activity at the micromolar range in HL 60 cells. Liscovitch *et al.*, (1992) also showed that increases in Ca^{2+} concentration inhibit GTPγS stimulated PLD activity in NG108-15 cells. In addition, Mg^{++}ATP potentiated the GTPγS effect in the neural derived NG108-15 cells, implicating the involvement of some type of kinase that augments the GTPγS-dependent stimulation. Addition of NEM (5 mM) resulted in complete inhibition of PLD.

Substitution of PIP_2 by PS in the substrate liposome also suggested that fibroblast PLD had an absolute requirement for this particular inositol-phospholipid rather than a non-specific requirement for a negative charge. In rat 1 fibroblasts, PLD activity was optimum when PIP_2 was presented in the substrate liposome at 12 μM. Higher concentrations (24 μM) decreased the activity probably by either disrupting the liposome or by blocking accessibility of the enzyme to the substrate. How PIP_2 confers its effect on the GTPγS-dependent stimulation of PLD remains to be elucidated. One possibility could be that PLD contains a binding site for PIP_2, which could control the conformational state of the protein. Several proteins have been shown to bind PIP_2, including profilin, F-actin, vinculin, α-actinin, and gelsolin. In addition PLD could also contain a pleckstrin homology (PH) domain which could mediate protein-protein interactions like the SH2 and SH3 domains. Harlan *et al.*, (1994) used a centrifugation assay to show that a number of

PH domains associate specifically with vesicles containing PIP_2. These possibilities could only be tested once the complete sequence of the purified isoform(s) of GTPγS-sensitive PLD has been obtained. The data presented in the Results section also suggested that ARF is an activator of PLD. ARF contains a highly specific binding site for PIP_2 (Boman *et al.*, 1995) implying that this phospholipid can modulate the activity of this G-protein. In fact, PIP_2 promoted the dissociation of GDP and stabilized the nucleotide free form of ARF (Terui *et al.*, 1994). PIP_2 could also play a role in the termination of the activated (GTP bound) state of ARF since ARF GAP activity is also highly dependent on PIP_2 (Randazzo *et al.*, 1994). PIP_2 is produced by phosphorylation of PI-4-P by phosphatidylinositol 4-phosphate 5-kinase. Chong *et al.* (1994) showed that RhoA regulates this activity and therefore the cellular levels of PIP_2. Since RhoA also regulates the formation of actin stress fibers and focal adhesions (Ridley and Hall, 1992), the authors (Chong *et al.*) (1994) suggested that PIP_2 could mediate the effects of RhoA on the cytoskeleton. In light of this information we substituted PIP_2 by PIP and asked the question: could the PLD activity be reconstituted in the presence of PIP, ATP and small molecular G proteins? Interestingly, RhoA or ARF alone were not able to recover PLD activity, but when both were present in the same assay about 50% of the activity, which was observed when PIP_2 was present, was recovered in a synergistic fashion. We think that the recovered PLD activity was due to newly produced PIP_2 although we did not measure cellular PIP_2 levels before and after the addition of ARF and RhoA. We cannot be sure that the observed synergism will hold true at higher concentrations of RhoA, since we could not saturate the system with this protein. Pertile *et al.* (1995) also showed that inhibition of PIP_2 synthesis potently inhibited PLD activation in permeabilized U937 cells which further suggests the importance of PIP_2 as a cofactor of PLD activity. Therefore it is possible that changes in PIP_2 concentrations in the micro environment of PLD and/or ARF, RhoA, RhoA GAP, and ARF GAP could lead to a tightly regulated stimulation of PA production through PLD.

More than 50% of the GTPγS stimulated activity was lost when GDPβS (3 mM) was present in the assays, suggesting that some of the observed PLD activity was independent from the action of a G-protein, or that even at this high concentration of GDPβS some proportion of ARF was still bound to GTPγS. The presence of the peptide containing the N-terminal sequence of ARF was sufficient for a complete inhibition of the GTPγS dependent PLD activity, providing strong evidence that ARF is responsible for the observed increase in PLD activity. ARF proteins require an intact amino terminus for full activity. Deletion of the N-terminal 13 or 17 amino acids renders ARF proteins inactive as cofactors for cholera toxin catalyzed ADP-ribosylation of G_S (Kahn *et al.*, 1992), without affecting their ability to bind GTP. In addition these mutant proteins also have decreased affinity for ARF GTPase-activating protein (Randazzo *et al.*, 1994). Further experiments will be required to find out if ARF interacts directly with PLD and if so, what the role that the N-terminal part of ARF plays in that interaction.

To prove conclusively that the GTPγS-dependent PLD activity observed in the presence of membranes plus cytosol is mediated through ARF, we purified recombinant, epitope-tagged ARF that was produced in Sf 9 cells. SDS-PAGE analysis showed that ARF was purified to near-homogeneity. Two doublets with apparent molecular weights of 19 and 23 kDa respectively were obtained after Coomassie staining. This altered electrophoretic mobility has also been observed in preparations of epitope tagged Ras proteins and has been attributed to farnesylation (post translational modification), with the modified version of the protein running at a lower molecular weight (Porfiri *et al.*, 1994). A simple way to prove that the band of lower apparent molecular weight is the myristoylated form of ARF would be to use radioactive myristate during the culture of Sf 9 cells and perform SDS-PAGE followed by autoradiography. Franco *et al.* (1995) also reported that the myristoylated form of ARF has higher electrophoretic mobility. Since myristoylation can add about 200 Da to a protein's apparent molecular weight, it seems counterintuitive that the processed protein runs further than its non-myristoylated counterpart, especially under denaturing conditions. Western blot analysis with a monoclonal antibody against ARF revealed strong cross-reactivity with both bands identifying the purified product as ARF. The last step in the characterization of purified ARF involved assaying its ability to stimulate PLD in the presence of GTPγS. Indeed, we were able to show that purified ARF could substitute the cytosolic requirement for GTPγS dependent activation of PLD from HL 60 membranes. Furthermore, the myristoylated form of recombinant ARF was a better activator of PLD in both 3T3 and HL 60 cells. Myristoylation of ARF occurs at a glycine residue residing at the amino terminus domain of the protein (Donaldson *et al.*, 1991), and appears to enhance greatly the ability of the protein to associate with membranes upon binding to GTP (Donaldson *et al.*, 1992). Recently Randazzo *et al.*, (1995) provided evidence suggesting that N-myristoylation favored the activation of ARF since this form had a greater affinity for GTPγS in the presence of phospholipids.

In light of the report presented by Malcolm *et al.* (1994) where stimulation of rat liver PLD by GTPγS involved a small molecular G protein of the Rho family, we prepared recombinant RhoA protein from a bacterial source. We used the purified product to test its ability to activate PLD using the cell free assay system. We observed a 1.6-fold activation of PLD from HL 60 membranes but no significant activation of PLD from 3T3 membranes in the presence of GTPγS. Addition of both recombinant ARF and RhoA to the assay had an additive effect to the activation of PLD, although Sidiqqi *et al.*, (1995) and Singer *et al.*, (1995) observed synergism under the same conditions. Our inability to also show synergism is probably due to our limited availability of recombinant proteins.

The modest activation of PLD in the presence of RhoA does not preclude the possibility that higher concentrations of recombinant RhoA could yield larger activation of PLD in both cell systems. So far we were unable to test for that, since we were hindered by the amount of purified protein we could obtain from our bacterial source. There is the possibility that at saturating levels, RhoA could stimulate PLD on its own, without the synergy of ARF.

It is also plausible that different isoforms of PLD are regulated by different mechanisms that could require the presence of one or the concerted action of multiple G proteins. Siddiqi *et al.* (1995) reported that GTPγS could stimulate membrane-bound PLD in HL 60 cells with no requirement of addition of cytosolic proteins, implicating the involvement of a G-protein other than ARF which resided in the membrane. Further evidence suggested to them, that RhoA is the activator of membrane-bound PLD in HL 60 cells with cytosolic PLD responding better to ARF. Moreover the cytosolic form of the enzyme was able to metabolize more readily endogenously labeled substrate and the membrane-bound form exogenous substrate in the form of a liposome. Their GTPγS stimulation of PLD (5-fold) in the absence of cytosol in HL 60 cells with exogenous substrate, is in contrast not only with the results presented in this thesis but also the ones reported by Brown *et al.* (1993) and Cockcroft *et al.* (1994). There is no doubt that RhoA also regulates PLD activity; what is not yet known is the mechanism by which PLD activity is regulated by small molecular G proteins.

Recently Gomez *et al.* (1994) showed that C_2- and C_6-ceramides inhibited the stimulation of PLD activity by phosphatidate and lysophosphatidate and a variety of growth factors in intact rat fibroblasts. In addition these cell-permeable ceramides inhibited stimulation of PLD activity by GTPγS in permeabilized fibroblasts. Using the cell free assay we also showed, for the first time, that C_2- but not C_6-ceramide reduced by about half the stimulation of membrane-bound PLD observed in the presence of ARF, RhoA, and the combination of the two in both HL 60 and 3T3 cells. Even though we tried to present ceramides to the assay in a number of different ways, we were unable to reproduce the inhibition of PLD activity that was seen with C_6-ceramide in the Gomez *et al.* (1994) report. Since we encountered solubility problems with this particular compound, it could be possible that C_6-ceramide never reached an inhibitory concentration in the assay.

Ceramides are the immediate products of sphingomyelinase action on sphingomyelin. Over the last few years increasing evidence has supported the notion that ceramides possess second messenger capacity as growth suppressors. Tumor necrosis factor-α and interleukin-1β can rapidly activate a neutral sphingomyelinase activity which generates a prolonged accumulation of ceramide which leads to programmed cell death (Hannun *et al.*, 1994). A number of potential intracellular targets for ceramide have been identified including a protein phosphatase (Obeid *et al.*, 1993), a membrane associated protein kinase (Goldkorn *et al.*, 1991) and the mitogen activated protein kinase cascade (Raines *et al.*, 1993).

The mechanism by which ceramides inhibit the G protein mediated stimulation of PLD activity in our system is not yet known. Ceramide could execute its potential inhibitory effects in a number of different ways. An obvious one would be to modulate the nucleotide state of either ARF or RhoA by stimulating, for example, the ARF- or RhoA-GAP activity thus terminating the active state of these G proteins. Alternatively, ceramides may have a direct effect on these proteins promoting conformational changes that impede nucleotide exchange or binding. Lastly the possibility that ceramides act upon PLD itself should not be overlooked; this can only be explored once PLD is purified. One aspect of the mode of action of ceramide has been uncovered by Jones *et al.* (1995) who showed that C_2-ceramide inhibits both the agonist stimulated translocation of PKC_{α}, and the resulting activation of PLD, although the authors could not exclude the possibility that ceramide could also inhibit G protein and/or PLD directly.

The PLD pathway has attracted the attention of many researchers worldwide. As more evidence accumulates about this enzymatic activity, the physiological role of PLD will become more clear. It is becoming apparent that PLD exists in multiple isoforms with different subcellular localizations and to say the least a complex regulation. PLD can be stimulated by a variety of agonists which in turn activate signaling cascades ranging from receptor tyrosine kinases, to PKC, and heterotrimeric and monomeric G proteins. In addition a number of other cytoplasmic (e.g.. ARF, a 50-kDa cytosolic factor, Lambeth *et al.*, 1994, etc.) and or membrane bound factors (RhoA) are also involved in modulation of PLD activity, suggesting that different isoforms of PLD could play important roles in several processes. It is possible that PLD is involved in a variety of physiological functions that take place in different areas of the cell, and is regulated by unique signal transduction pathways. PLD could participate in membrane traffic since ARF has been shown to regulate formation and or fusion of transport vesicles. On the other hand, PLD could also be involved in cell shape changes, cellular adhesion, and differentiation which are modulated by RhoA and other proteins of the Rho family. On the other hand PLD could serve as the integration point of parallel pathways leading to major changes in PA levels, which in turn elicits a number of cellular responses (Discussed in more detail in the Introduction).

Phosphatidate phosphohydrolase is responsible for the conversion of PA to DAG thus exchanging one potent second messenger (PA) for another (DAG). Martin *et al.*, (1993) showed that the NEM- insensitive form of PAP, PAP-2, is involved in signal transduction since the gross differences in PA levels between *ras* - and control fibroblasts were correlated to differences in PAP-2 activity between the two cell lines. In the control cells, the specific activity of PAP-2 increased with time in culture as the cells stop growing by becoming contact inhibited. The PA levels in the control fibroblasts were inversely related to the activity of PAP-2. In contrast, *ras* -transformed cells exhibited low specific activity of PAP-2 and higher PA levels with respect to time in culture and remained in the log phase with respect to growth curve. The NEM-sensitive form of PAP, PAP-1, also followed the same activity trend that PAP-2 exhibited in both cell lines. The decreased activities of PAP-1 could contribute to the increased levels of PA in the transformed cells especially since we also discovered that PAP-1 in *ras* -transformed cells was unable to translocate to the membranes in response to oleate.

At the moment we are unable to explain the differences of activity and translocation profiles of PAP-1 between the two fibroblast cell lines since PAP-1 purification remains elusive. With the appropriate molecular tools, we could find out if differences in the localization phosphorylation and expression of the protein in control and *ras* -transformed fibroblasts , as well as the role, if any, of PAP-1 in signal transduction.

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